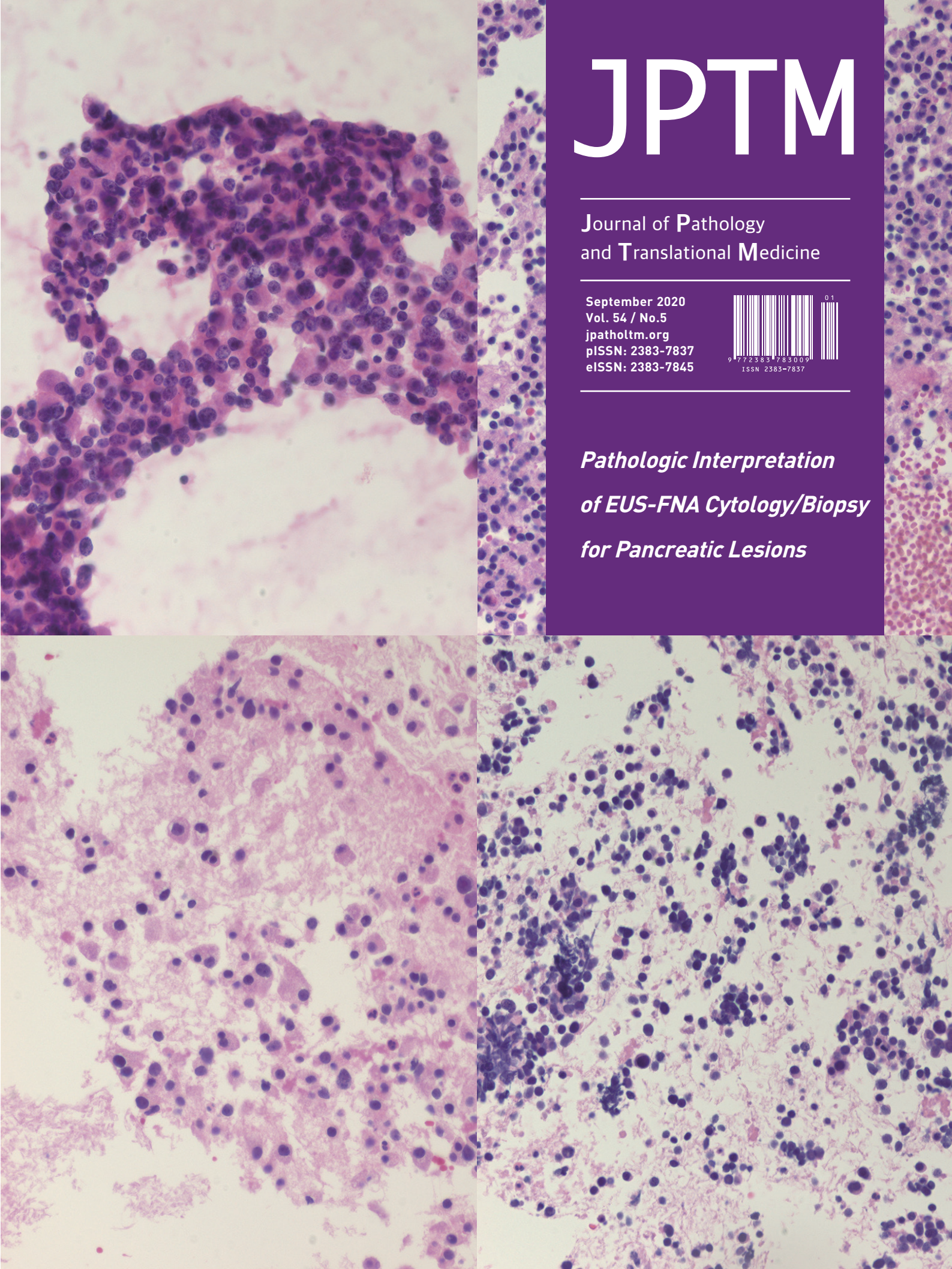




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*Pathologic Interpretation
of EUS-FNA Cytology/Biopsy
for Pancreatic Lesions*

Aims & Scope

The *Journal of Pathology and Translational Medicine* is an open venue for the rapid publication of major achievements in various fields of pathology, cytopathology, and biomedical and translational research. The Journal aims to share new insights into the molecular and cellular mechanisms of human diseases and to report major advances in both experimental and clinical medicine, with a particular emphasis on translational research. The investigations of human cells and tissues using high-dimensional biology techniques such as genomics and proteomics will be given a high priority. Articles on stem cell biology are also welcome. The categories of manuscript include original articles, review and perspective articles, case studies, brief case reports, and letters to the editor.

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Immune landscape and biomarkers for immuno-oncology in colorectal cancers

Jeong Mo Bae, Seung-Yeon Yoo, Jung Ho Kim, Gyeong Hoon Kang

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Recent advances in immuno-oncology have increased understanding of the tumor immune microenvironment (TIME), and clinical trials for immune checkpoint inhibitor treatment have shown remission and/or durable response in certain proportions of patients stratified by predictive biomarkers. The TIME in colorectal cancer (CRC) was initially evaluated several decades ago. The prognostic value of the immune response to tumors, including tumor-infiltrating lymphocytes, peritumoral lymphoid reaction, and Crohn's-like lymphoid reaction, has been well demonstrated. In this review, we describe the chronology of TIME research and review the up-to-date high-dimensional TIME landscape of CRC. We also summarize the clinical relevance of several biomarkers associated with immunotherapy in CRC, such as microsatellite instability, tumor mutational burden, *POLE/POLD* mutation, consensus molecular subtype, and programmed death-ligand 1 expression.

Key Words: Colorectal neoplasms; Tumor immune microenvironment; Immunotherapy; Microsatellite instability

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Colorectal cancer (CRC) is the second most commonly diagnosed cancer and the third leading cause of cancer-related death in Korea [1]. Because of rapid spread of colonoscopy screening, there was a general decrease in CRC until 2010. However, recent studies have reported that the decline in CRC incidence has reversed during the last few years, especially in middle-aged persons, and the occurrence of early-onset CRC has rapidly increased [2]. Most patients with CRC are diagnosed at an operable stage; however, approximately 20% of patients with stage III or high-risk stage II CRC relapse within 5 years after curative resection [3]. Moreover, the 5-year relative survival rate for metastatic CRC is 14% [2]. To improve clinical outcomes for patients with CRC, a more effective treatment modality is required to fulfill those unmet needs.

Cancer is fundamentally a genetic disease since the accumulation of mutations, fusions, and copy number alterations drives tumorigenesis. However, recent research on the tumor immune microenvironment (TIME) has revealed the importance of interactions between tumor cells and surrounding immune cells in tumorigenesis [4]. Immune checkpoint inhibitor (ICI) treat-

ment, such as anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibodies and anti-programmed death-1 (PD-1) antibodies, has shown marked clinical benefits in many types of cancer [5,6]. CRC also holds promise for cancer immunotherapy use, and the U.S. Food and Drug Administration (U.S. FDA) approved immunotherapeutic agents for microsatellite instability-high (MSI-H) CRC in 2017 (Fig. 1) [7,8].

In this review, we describe the landscape of the immune microenvironment in CRC and summarize the clinical usefulness of several suggested biomarkers in CRC immunotherapy.

IMMUNE LANDSCAPE OF COLORECTAL CANCERS

Historical use of microscopic evaluation of immune environment in CRCs

To our knowledge, Spratt and Spjut [9] published the first study on integrative histologic evaluation of CRCs in 1967. In that study, the authors evaluated histologic grade, mucinous elements, depth of invasion, characteristics of tumor border, lymph-

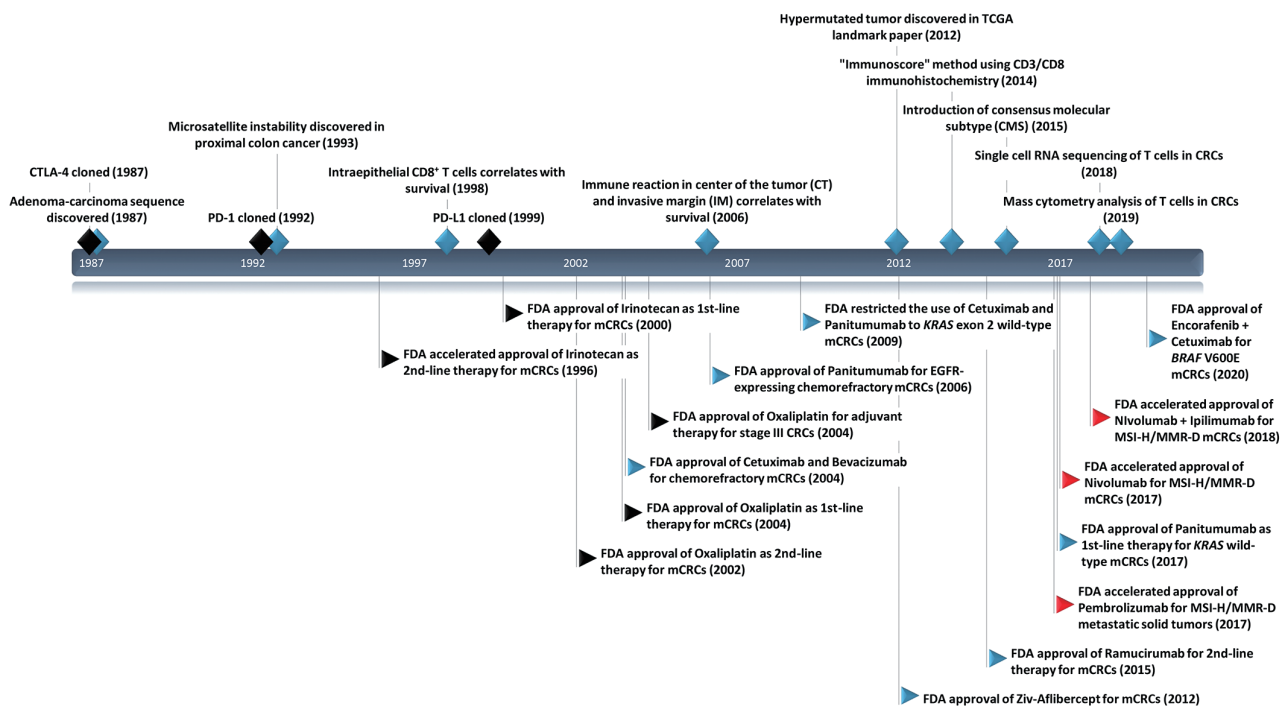


Fig. 1. Timeline with key milestones in immuno-oncology research and U.S. Food and Drug Administration (FDA)-approved anti-cancer therapy in colorectal cancers (CRCs). CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TCGA, The Cancer Genome Atlas; mCRC, metastatic colorectal cancer; MSI, microsatellite instability; MMR-D, mismatch repair deficiency; MSI-H, MSI-high. Black diamond, milestone events in general immuno-oncology research; blue diamond, milestone events in immuno-oncology research in CRCs; black flags, cytotoxic chemotherapy; blue flags, targeted therapy; and red flags, immunotherapy.

phatic/vascular/perineural invasion, and especially degree of inflammatory reaction around the tumor in 1,137 consecutive CRC cases. CRC with no inflammatory reaction showed shorter 5-year and 10-year survivals than CRC with moderate inflammatory reaction and abscess formation. Watt and House [10] evaluated peritumoral lymphoid cells in a semiquantitative manner, and Duke B tumors with recurrence or death during follow-up showed a tendency toward minimal lymphocytic infiltration. Pihl et al. [11] reported the association of perivascular lymphoid cuffing in the muscularis propria or subserosa with favorable disease-free survival (DFS) in 134 Duke B CRCs. Perivascular lymphoid cuffing has been named “Crohn’s-like lymphoid reaction,” and is now referred to as an ectopic or tertiary lymphoid structure [12]. Naito et al. [13] evaluated intraepithelial tumor-infiltrating CD8⁺ T cells using immunohistochemistry and found that increased intraepithelial CD8⁺ cell infiltration was associated with lower Duke stage and better survival. Ogino et al. [14] reported that the overall lymphocytic reaction, which combines the degree of Crohn’s-like lymphoid reaction, peritumoral reaction, intratumoral periglandular reaction, and tumor-infiltrating lymphocytes (TILs), is a prognostic marker independent of clinicopathologic and molecular characteristics.

Quantitative evaluation of TIME using digital pathology

Previous studies evaluating the immune microenvironment in CRCs depended on manual inspection of glass slides using a light microscope. Visual assessment of immune cells and stroma is labor-intensive and has limited objectivity. Most studies enumerated immune cells in hotspot areas, while some studies used semiquantitative methods [15]. Recent advancements in virtual slide scanners and machine learning algorithms have enabled objective quantification of the tumor microenvironment at the whole slide level.

The immunoscore developed by Jérôme Galon is the most well-known digital pathology-based approach for evaluation of TILs. In 2006, Galon et al. [16] showed that an increased number of total T cells (CD3⁺) and resident memory T cells (CD45RO⁺) in the center of the tumor (CT) and at the invasive margin (IM) was an independent marker of better DFS in CRCs. In 2014, they proposed the “Immunoscore” method based on enumeration of CD3⁺ and CD8⁺ T cells in the CT and IM regions of tumors (Fig. 2) [17]. Immunoscore assay shows superior prognostic value compared with microsatellite instability and has prognostic value in both primary tissues and metastatic tissues [18,19]. To ensure robust enumeration of TILs, the researchers

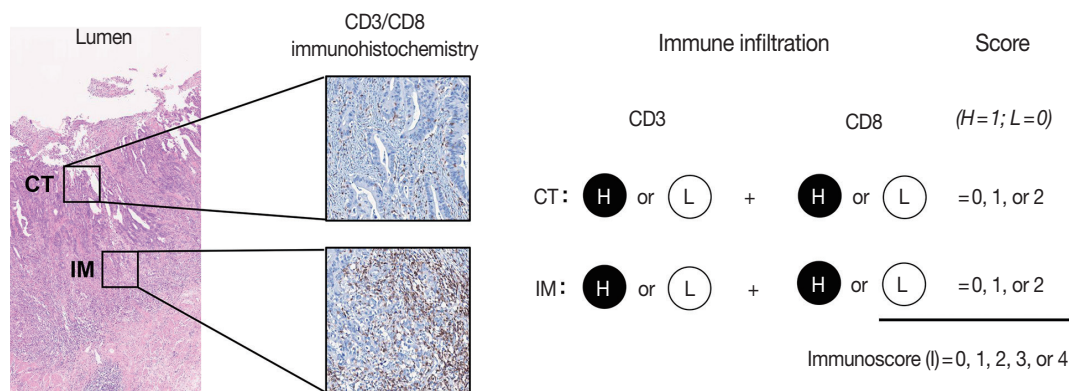


Fig. 2. Calculation method of Immunoscore (adapted from Anitei et al. [17]). CT, center of tumor; IM, invasive margin; H, high; and L, low.

developed an in vitro diagnostic immunoscore assay for clinical use. The international immunoscore consortium consisted of 14 institutions in 13 countries and performed a validation study for the immunoscore assay using 2,681 stage I–III colon cancers [20]. Using standardized immunohistochemistry protocols and image analysis software, the immunoscore assay showed high reproducibility between institutions. The consortium categorized CRC cases as low immunoscore group (0–25 percentile), intermediate immunoscore group (25–70 percentile), and high immunoscore group (70–100 percentile). Patients with high immunoscore showed longer time to recurrence compared to patients with low or intermediate immunoscore independent of age, sex, stage, microsatellite instability (MSI), and other known prognostic factors. Moreover, patients with high immunoscore showed significantly lower risk of recurrence compared to patients with low immunoscore in stage II colon cancers.

Reichling et al. [21] performed whole-slide imaging analysis of 1,018 stage III colon cancers in the PETACC08 study. They developed software to detect colon cancer, normal mucosa, stroma, and immune cells on CD3- and CD8-stained slides. In their study, the stromal area in IM and CT (originally, tumor core in their article) showed strong positive correlation. A higher proportion of stromal area in the tumor was associated with poor relapse-free survival (RFS), and an increased proportion of stromal area in the IM showed the highest hazard ratio for RFS compared with the stromal area in the CT or total stromal area. The researchers tested the prognostic role of the four immune variables (mean values of each tumor tile for each slide), CD3⁺ IM, CD3⁺ CT, CD8⁺ IM, and CD8⁺ CT. High CD3⁺ IM, CD3⁺ CT, and CD8⁺ CT were significantly associated with superior clinical outcomes. The classical “Immunoscore” showed similar performance to the CD3⁺ CT variable in predicting clinical outcome. The researchers developed the “DGMate” score de-

rived from 127 parameters extracted from image analysis and the “DGMuneS” score by combining CD3⁺ CT, stromal area in the IM, and “DGMate” score. The “DGMuneS” score slightly outperformed the “Immunoscore” in predicting clinical outcome.

Nearchou et al. [15] evaluated tumor bud (TB), CD3⁺ TILs, and CD8⁺ TILs on the same section of stage II CRC tissues using multiplex immunofluorescence staining. They found that a higher density of CD3⁺ T cells at the IM and CD8⁺ T cells at the IM and in whole tumor sections (WTSs) were significantly correlated with lower TB number. In survival analysis using least absolute shrinkage and selection operator penalized Cox proportional hazard regression, four morphologic features, including CD3⁺ T cell density in WTSs, mean CD3⁺CD8⁺ T cell number within 50 μm of TBs, TB number, and CD8⁺ T cell density in the CT, had significant predictive value for disease-specific survival. After eliminating the least significant features, the authors proposed the “Tumor Bud-Immuno Spatial Index (TBISI)” using CD3⁺ T cell density in WTSs, mean CD3⁺CD8⁺ T cell number within 50 μm of TBs, and TB numbers, which could predict disease-specific survival.

We recently analyzed CD3⁺ TILs, CD8⁺ TILs, and the tumor-stroma ratio (TSR) in 886 stage III or high-risk stage II CRCs using whole-slide imaging [22]. Clustering analysis using 197 parameters extracted from image analysis classified CRCs into five clusters. Strikingly, the five clusters showed similar clinicopathologic and molecular characteristics with the consensus molecular subtype (CMS) classification [23]. In detail, cluster 1 was characterized by highest TIL density, enrichment of MSI-H tumors, and CpG island methylator phenotype-high (CIMP-H) tumors (features of CMS1). Cluster 2 was characterized by lower TSR, distal colorectal locations, and retained intestinal differentiation (features of CMS2). Cluster 3 was characterized by the highest CD8/CD3 ratio and prominent mucin production (fea-

tures of CMS3). Cluster 4 was characterized by the lowest TIL density and highest TSR (features of CMS4). Cluster 5 showed intermediate TIME characteristics, and this cluster was similar to tumors with mixed/indeterminate features in the original CMS study. This similarity highlights the clear association of the tumor transcriptome with the immune microenvironment [24]. Similar to the original CMS classification, cluster 4 in our study showed poor 5-year RFS in two independent datasets.

High-dimensional analysis of immune landscape of CRCs

Recent advancements in RNA sequencing, proteomics, and single-cell technologies have dramatically increased our understanding of the TIME. Deconvolution algorithms such as CIBERSORT [25], xCELL [26], and microenvironment cell populations-counter (MCP-counter) [27] for bulk RNA sequencing are useful tools for transcriptome data [28]. Because there are many publicly available transcriptome data for CRCs combined with genetic and clinicopathologic data from different datasets, TIME characteristics of each molecular or pathologic subtype can be identified. Using gene expression signatures, these algorithms can identify the cellular fractions of 6 to 64 immune and nonimmune cells. Mass cytometry provides high-dimensional protein-based cellular data for up to 40 antibodies at an individual cell level [29]. Combining cytometry and the time-of-flight method using lanthanide metal ion-tagged antibodies, mass cytometry provides high-dimensional data with low background noise. Single-cell RNA sequencing (scRNA-seq) provides unbiased profiling of immune cells without prior gene selection [30]. scRNA-seq enables classification of different subsets and identification of novel markers or regulators for each subset. Both mass cytometry and scRNA-seq used in an in-situ manner provide a more comprehensive TIME landscape by preserving spatial information [31,32].

Xiong et al. [33] analyzed the proportion of 22 cell types in bulk transcriptome data from 2,306 patients with CRC (644 from The Cancer Genome Atlas [TCGA] RNA-sequencing data and 1,662 from Gene Expression Omnibus expression microarray data) using CIBERSORT. Tumor tissues showed more M0 and M1 macrophages, resting natural killer (NK) cells, plasma cells, and memory and activated CD4⁺ T cells along with fewer resting mast cells and M2 macrophages than normal tissues. In survival analysis, M1 macrophages and activated dendritic cells were significantly associated with improved outcome, whereas eosinophils, neutrophils, and M2 macrophages were associated with poorer outcomes. Marisa et al. [34] quantified immune cell infiltration in transcriptome data using an

MCP-counter. There was a strong positive association between immune checkpoint expression with infiltration of certain lymphoid (NK cells, T cells, and cytotoxic cells) and myeloid cells, whereas B cells, fibroblasts, vessels, and granulocytes showed little or no association with immune checkpoint expression.

Zhang et al. [35] performed scRNA-seq and T cell receptor (TCR) tracking to analyze distinct functions and clonalities among 11,138 T cells from 12 patients with CRC. Using the t-stochastic neighbor embedding method, the authors found a total of eight CD8⁺ and 12 CD4⁺ T cell clusters. Within CD8⁺ T cells, naïve T cells, central memory T cells, and recently activated effector memory T (T_{EMRA}) cells were enriched in blood, whereas exhausted T (T_{EX}) cells were specifically enriched in tumors. Resident memory T cells were predominantly found in normal mucosa. Among CD4⁺ subtypes, naïve and effector-like cells were enriched in blood. Follicular helper T cells were enriched in normal mucosa, whereas two IFNG⁺ Th1-cell-like subsets and Th17 cells were enriched in tumors. In clonality analysis, CD8⁺ T_{EX} cells and T_{EMRA} cells showed the highest degree of clonal expansion. Among CD4⁺ T cells, most tumor-infiltrating regulatory T (Treg) cell clones showed clonal exclusivity, whereas certain Treg cell clones were developmentally linked to several helper T cell clones. The researchers also found that CXCL13⁺BHLHE40⁺ Th1-like cells were abundant in MSI tumors, whereas microsatellite-stable (MSS) tumors were moderately enriched with T_H17 cells. BHLHE40 is expressed in T cells via TCR stimulation, which positively regulates granulocyte-macrophage colony-stimulating factor and IFN- γ production [36,37]. The authors speculated that enrichment of CXCL13⁺BHLHE40⁺IFNG⁺ Th1-like cells might be one cause of a favorable response to immunotherapy in patients with MSI CRC. Recently, anti-CD40 agonist treatment was reported to increase BHLHE40⁺ Th1-like cells in a MC38 syngeneic mouse tumor model. This finding suggests crosstalk of tumor-associated BHLHE40⁺ Th1-like cells and conventional type 1 dendritic cells [38].

De Vries et al. [39] performed single-cell mass cytometry using 36 immune cell markers in 35 CRC tissues, 26 tumor-associated lymph nodes, 17 healthy mucosae, and 19 peripheral blood samples from 31 patients with CRC. Clustering analysis of CD8⁺/ $\gamma\delta$ T cells revealed that activated (HLA-DR⁺CD38⁺PD-1⁺) and tissue-resident (CD103⁺CD69⁺) phenotypes were enriched in tumor tissue compared with other tissues. Clustering analysis of CD4⁺ T cells showed that inducible T-cell co-stimulator (ICOS)⁺CD27⁻ cells were enriched in tumor tissues, and these cells showed a regulatory-like phenotype overexpressing

FOXP3. In innate lymphocyte populations, Lin[−]CD7⁺CD127[−]CD56⁺CD45RO⁺ cells were enriched in tumor tissues, accounting for up to 80% of the innate lymphoid compartment. This subset showed a tissue-resident (CD103⁺CD69⁺) phenotype and displayed cytotoxic activity. Moreover, this cell population was abundant in mismatch repair (MMR)-deficient tumors. Norton et al. [40] showed that B lymphocyte-induced maturation protein-1 (BLIMP1)⁺ Treg cells were significantly enriched in tumor tissues compared with normal mucosa. The enrichment of ICOS, CD45RO, PD-1, programmed death-ligand 1 (PD-L1), lymphocyte-activation gene 3 (LAG-3), CTLA-4, and T-cell immunoglobulin mucin-3 (TIM-3) on BLIMP-1⁺ regulatory T cells suggested that BLIMP-1⁺ Treg cells have a more activated phenotype than conventional Treg cells and may play a role in the antitumor immune response. Di et al. [41] also found that exhausted T cells (PD-1⁺CD38⁺HLA-DR⁺CCR7⁺CD127[−]) and regulatory T cells (CD4⁺CD25⁺CD127[−]) were increased in tumor tissues. Moreover, they found that CD8⁺CD28[−] immunosenescent T cells with impaired proliferation capacity were the most abundant T cell population in colorectal tumors.

BIOMARKERS ASSOCIATED WITH IMMUNE MICROENVIRONMENT AND IMMUNOTHERAPY

Microsatellite instability/mismatch repair-deficiency

Germline mutation of genes encoding MMR enzymes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) or promoter hypermethylation of the *bMLH1* gene causes MMR deficiency (MMR-D) [42]. MMR-D causes numerous frameshift mutations that result in increased neoantigen production [43]. Increased neoantigen production causes vigorous immune reactions in MSI-H CRCs, such as increased TIL infiltration, peritumoral lymphocytic infiltration, and Crohn's-like lymphoid reactions. How MSI-H CRCs persist in a hostile immune microenvironment is of great interest. Llosa et al. [44] showed that although MSI-H CRCs showed high infiltration by CD8⁺ cytotoxic T lymphocytes and Th1 cells, these tumors showed upregulated expression of immune checkpoint molecules, including PD-1, PD-L1, CTLA-4, LAG-3, and indoleamine 2,3-dioxygenase. This finding suggested that MSI-H CRC may be a good candidate for ICI treatment. MSI-H/MMR-D CRCs are more responsive to PD-1 blockade than MSS/MMR-proficient CRCs [7]. Because MSI/MMR status showed predictive value in other extracolonic cancers, MSI/MMR status became a tissue-agnostic predictive biomarker for ICI treatment [45]. The reported response rate is 28%–52%, and the disease control rate is 51%–82% for ICI in MSI-H/MMR-D

CRCs [7,8,46]. Currently, the anti-PD-1 inhibitor pembrolizumab can be used as third-line therapy for MSI-H/MMR-D CRCs by off-label use after agreement by a multi-disciplinary team in designated institutions in Korea.

Tumor mutational burden

Tumor mutational burden (TMB) is a measure of the total amount of somatic coding mutations in a tumor, and it is considered an emerging biomarker for ICI treatment [47]. Initially, the concept of TMB was derived from whole exome sequencing (WES); however, many studies revealed that TMB calculated from targeted next generation sequencing panels showed clinically compatible results with WES [48]. TMB values should be interpreted cautiously because the calculation formula for TMB varies among different panels, and the cut-off for TMB-high (TMB-H) status differs among clinical trials and tumor types [49].

TCGA consortium reported that 15.9% of 549 CRCs showed hypermutation ($\geq 10/\text{Mb}$) using WES [50]. However, Parikh et al. [51] reported that TMB-H was observed in 4.9% of 12,569 CRCs using a cut-off of $\geq 20/\text{Mb}$ analyzed by the FoundationOne panel. Although MSI is a proven predictive marker for ICI treatment, patients can be stratified further by TMB status. Schrock et al. [46] recently reported that patients showing objective response had higher TMB (median, 54/Mb; range, 31 to 91/Mb) than non-responders (median, 29/Mb; range, 13 to 37/Mb) in 22 patients with metastatic MSI-H CRC treated with anti-PD-1 or anti-PD-L1 treatment [46]. Lee et al. [52] reported that TMB was a prognostic marker of better RFS in stage III or high-risk stage II CRCs treated with oxaliplatin-based adjuvant chemotherapy, independent of MSI status.

Three-quarters of TMB-H CRCs are MSI-H, and the remaining one-quarter are MSS with somatic mutations in proofreading genes, mainly polymerase ϵ (*POLE*) and polymerase δ (*POLD*) [53]. In mutation analysis, MSI-H tumors show an insertion-deletion (indel)-predominant pattern, while *POLE* mutants show a single nucleotide variation-predominant pattern [54,55]. Domingo et al. [56] analyzed the frequency of somatic *POLE* mutations in 6,517 CRCs, and *POLE* mutations were detected in 1% of CRCs. CRCs with *POLE* mutations are associated with younger age at diagnosis, male sex, and proximal location [55,56]. Both *POLE* mutation and MSI-H/MMR-D status were associated with reduced risk of recurrence in a retrospective study [56].

There are limited data about the predictive value of *POLE* mutations in ICI treatment. In Wang et al.'s study [57], three

patients harboring pathogenic *POLE* mutations received ICI treatment, and only one patient showed complete remission. Silberman et al. [58] reported a case of complete and sustained response to anti-PD-1 treatment in a patient with metastatic CRCs harboring a pathogenic p.Val411Leu *POLE* mutation.

Consensus molecular subtype

Several investigators have suggested gene expression-based CRC classifications [59-61]. Due to the similarity among different classifications, the international CRC Subtyping Consortium proposed a unified transcriptomic classification, which was named the “consensus molecular subtype” (CMS) classification [23]. CMS1 (14%, MSI immune subtype) is characterized by MSI-H and CIMP-H statuses in addition to hypermutation and *BRAF* mutation. CMS1 also shows high immune infiltration and activation. CMS2 (37%, canonical subtype) is characterized by somatic copy number alterations and WNT and MYC activation. CMS3 (13%, metabolic subtype) shows metabolic deregulation and is associated with *KRAS* mutations. CMS4 (23%, mesenchymal subtype) is characterized by stromal infiltration, transforming growth factor β (TGF- β) activation, and angiogenesis. In the original CMS paper, CMS1 was associated with worse survival after relapse, while CMS4 was associated with worse RFS and overall survival.

By applying deconvolution algorithms to bulk transcriptome data, Becht et al. [24] and Karpinski et al. [62] evaluated the cellular composition of each CMS subtype in CRCs. In Becht et al.'s study [24] using an MCP-counter, CMS1 and CMS4 showed high expression of lymphoid and myeloid cell-specific genes. However, CMS1 exhibited high infiltration by CD8⁺ cytotoxic T cells and NK cells, whereas CMS4 showed high expression of fibroblastic and endothelial cells [24]. Karpinski et al. [62] measured the proportion of 22 immune cell subtypes in 1,597 CRCs using CIBERSORT. CMS1 showed enrichment of leukocytes related to adaptive immunity (follicular helper T cells, memory activated helper T cells, and cytotoxic T cells) and innate immunity (activated NK cells, $\gamma\delta$ T cells, M1 macrophages, activated dendritic cells, activated mast cells, and neutrophils). In addition, CMS1 showed depletion of Treg cells. CMS2 showed enrichment of helper T cells and memory B cells. CMS3 showed low levels of immune activation that manifested as high levels of resting memory helper T cells, naïve B cells, and low levels of macrophages, neutrophils, and activated helper T cells. Last, CMS4 was characterized by the highest proportions of leukocytes related to protumor activity (eosinophils, monocytes, M2 macrophages, resting dendritic cells, and regulatory

T cells).

Soldevilla et al. [63] evaluated the distribution of six immune subtypes proposed by Thorsson et al. [64] among each CMS subtype in TCGA samples. In a total of 625 samples, the C1 *wound healing* subtype (77%) was the most predominant, followed by the C2 *INF- γ dominant* subtype (17%). The C1 *wound healing* subtype was predominant in the CMS2 subtype (92%) but less common in the CMS1 subtype (46%). In contrast, the C2 *INF- γ dominant* subtype was the most common immune subtype in CMS1 (53%) and was underrepresented in CMS2 (8%). CMS3 showed a higher frequency of the C3 *inflammatory* subtype (7%) and the C4 *lymphocyte-depleted* subtype (4%) than the other CMSs. The C6 *TGF- β dominant* subtype was exclusively observed in CMS4.

Marisa et al. [34] evaluated whether the prognostic value of immune gene expression varies according to CMS. The expression of genes associated with Th1 cells, cytotoxic T cells, and cytotoxicity was predictive of better prognosis in MSS tumors from CMS2 and CMS3. In contrast, they had no prognostic relevance in CMS1 and CMS4. A brief summary of genomic and immunologic characteristics of each CMS subtype is shown in Table 1.

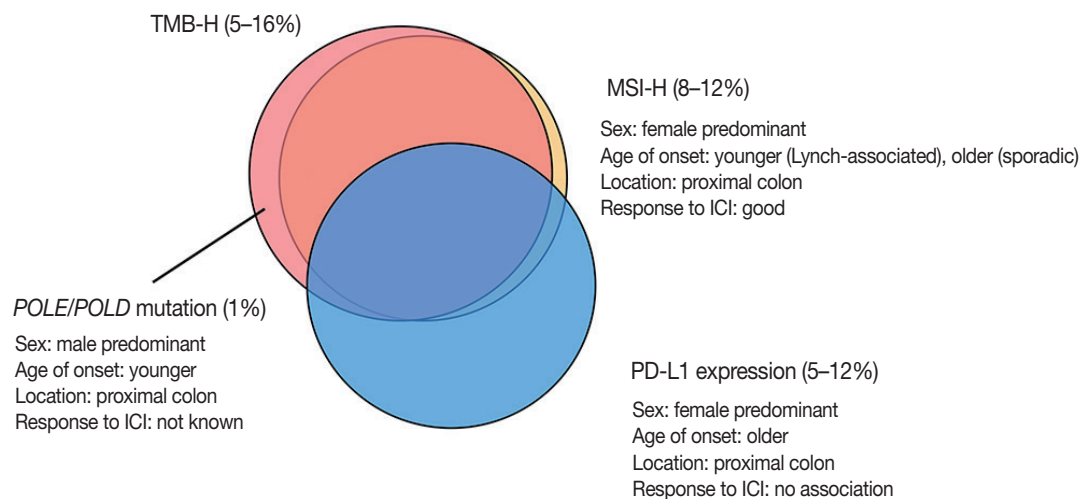
PD-L1 expression

Several studies evaluated the clinicopathologic characteristics of PD-L1 expression in CRCs. The frequency of PD-L1 overexpression in tumor cells is approximately 5% to 12% in all CRCs (1 to 4% in MSS CRCs and 18% to 45% in MSI-H CRCs) [65-69]. PD-L1 overexpression is associated with female sex, right-sided colon occurrence, poor differentiation, solid or medullary histology, increased number of TILs, and *BRAF* V600E mutation. Although a recent meta-analysis showed that PD-L1 overexpression in tumor cells was significantly associated with poor overall survival and decreased DFS, the prognostic value of PD-L1 overexpression differed according to MSI status [70]. Lee et al. [66] showed that the association of decreased recurrence-free survival with PD-L1 overexpression was only observed in MSI-H CRCs. In Marisa et al.'s transcriptome analysis [34], there was a strong association of immune checkpoint expression with infiltration of specific lymphoid (NK cells, T cells, and cytotoxic cells) and myeloid cells, whereas B cells, fibroblasts, vessels, and granulocytes showed little or no association with immune checkpoint expression. Patients with MSI-H CRC with high immune checkpoint expression, including high expression of PD-L1, showed significantly poorer overall survival compared to patients with MSI-H CRC and low immune

Table 1. Genomic and immunologic characteristics of consensus molecular subtype

	CMS1 MSI immune (14%)	CMS2 canonical (37%)	CMS3 metabolic (13%)	CMS4 mesenchymal (23%)
Genomic feature	MSI CIMP-positive Hypermutation SCNA-low	MSS CIMP-negative SCNA-high	MSS CIMP-low SCNA-intermediate	MSS CIMP-negative SCNA-high
Expression signature	Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF- β activation, angiogenesis
Immune-subtype	Immune activated	Immune desert	Immune excluded	Immune inflamed
Immune cell infiltration	CD8 ⁺ TIL $\uparrow$ Macrophages $\uparrow$ NK cells $\uparrow$			MDSC $\uparrow$ Treg $\uparrow$ Th17 $\uparrow$
Immune gene expression	T cell chemotaxis and activation (CXCL9, CXCL10, CXCL16, IFN- γ) $\uparrow$ T cell-specific inhibition (PD-1, PD-L1, CTLA-4, LAG-3) $\uparrow$ MHC1 (HLA, B2M) $\uparrow$	MHC1 (HLA, B2M) $\downarrow$		Angiogenesis (VEGF) $\uparrow$ Immunosuppression (TGF- β) $\uparrow$ Complement $\uparrow$
Immune subtype by Thorsson et al. [63,64]	C2 INF- γ dominant $\uparrow\uparrow$	C1 wound healing $\uparrow\uparrow$	C3 inflammatory $\uparrow$ C4 lymphocyte depleted $\uparrow$	C6 TGF- β dominant $\uparrow$
TIME cluster by Yoo et al. [22]	Cluster 1, TIL $\uparrow\uparrow$	Cluster 2, TSR $\downarrow\downarrow$	Cluster 3, CD8/CD3 $\uparrow\uparrow$	Cluster 4, TSR $\uparrow\uparrow$

CMS, consensus molecular subtype; MSI, microsatellite instability; MSS, microsatellite stable; CIMP, CpG island methylator phenotype; SCNA, somatic copy number alteration; TIL, tumor-infiltrating lymphocytes; NK, natural killer; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; IFN, interferon; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; CTLA-4, cytotoxic T-lymphocyte antigen 4; LAG-3, lymphocyte-activation gene 3; TGF- β , transforming growth factor- β ; TSR, tumor-stroma ratio.

**Fig. 3.** Correlation of tumor mutational burden, microsatellite instability, *POLE/POLD* mutation, and programmed death-ligand 1 (PD-L1) expression in colorectal cancers. TMB-H, tumor mutational burden-high; MSI-H, microsatellite instability-high; ICI, immune checkpoint inhibitor.

checkpoint expression. However, immune checkpoint expression status did not influence the clinical outcome of patients with MSS CRC.

In addition, the frequency of PD-L1 expression in MSI-H CRCs differs by the etiology of MSI. In our previous study, MSI CRCs with *MLH1* methylation showed a higher frequency of PD-L1 expression (28.3%) than MSI CRCs without *MLH1* methylation (6.1%) [67]. Yamada et al. [71] reported similar

results, which showed increased PD-L1 expression in sporadic MSI-H CRCs (25.0%) compared to Lynch syndrome-associated CRCs (3.6%). The underlying mechanism of PD-L1 overexpression in sporadic MSI CRCs should be further investigated.

Although PD-L1 expression is considered a predictive marker for ICI treatment in several types of cancer, such as melanoma and non-small-cell lung cancer, currently available data have demonstrated that PD-L1 expression has no predictive value in

CRC with ICI treatment [7,8]. The discrepancy between PD-L1 expression and response to ICI treatment might be related to spatiotemporal variations in PD-L1 expression, the cut-off for PD-L1 positivity, or interobserver variations in interpretations. The correlations of each biomarker described above are summarized in Fig. 3.

CONCLUSION

Evaluation of the TIME in CRCs has advanced from semi-quantitative visual inspection to quantitative and high-dimensional approaches. Numerous clinical trials are ongoing to develop next-generation immune checkpoint drugs that target checkpoint modulators beyond PD-1/PD-L1, such as TIM-3, LAG-3, and T cell immunoreceptor with Ig and ITIM domains [72]. Combination immunotherapies, which combine cytotoxic chemotherapeutic agents or vascular endothelial growth factor inhibitors with ICIs, are also being investigated. The importance of comprehensive understanding of the TIME is constantly increasing in the era of immunotherapy. Moreover, numerous efforts are required to develop more precise biomarkers to classify responders to immunotherapy in CRC.

Ethics Statement

Not applicable.

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Conflicts of Interest

J.H.K. and G.H.K., contributing editors of the *Journal of Pathology and Translational Medicine*, were not involved in the editorial evaluation or decision to publish this article. All remaining authors have declared no conflicts of interest.

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Thyroid fine-needle aspiration cytology in Taiwan: a nationwide survey and literature update

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In Taiwan, thyroid fine-needle aspiration cytology is easily accessible and reliable for evaluating thyroid nodules. The sonographic pattern plays a major role and is the deciding factor for aspiration. We conducted a nationwide survey in 2017 and it revealed that 31% of laboratories had adopted The Bethesda System for Reporting Thyroid Cytopathology. There was a relatively high unsatisfactory rate (24.04%) and low rates of indeterminate diagnoses, including atypia of undetermined significance/follicular lesions of undetermined significance: 4.87%, and follicular neoplasm/suspicious for a follicular neoplasm: 0.35%. Moreover, the risks of malignancy in benign, atypia of undetermined significance, and suspicious for a follicular neoplasm were relatively high. These may reflect strict diagnostic criteria for indeterminate categories and better patient selection for surgery. Improvements in specimen sampling and continuing education programs are crucial. Newly-developed thyroid cytology technologies, such as immunocytochemistry, molecular testing, and computerized cytomorphometry, may further facilitate cytology diagnoses.

Key Words: Cytology; Fine-needle aspiration; Taiwan; Thyroid; Thyroidectomy

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Fine-needle aspiration (FNA) is minimally invasive and widely used for assessing thyroid nodules [1]. It is also a decisive procedure for evaluating thyroid nodules that require surgery or conservative management [2,3]. Although the Bethesda System is the most widely accepted diagnostic system for thyroid cytology, there are significant deviations in practices in different countries [4,5]. Our previous review summarized the history and the evolution of the thyroid FNA practice in Taiwan [6]. The aims of this article are to provide an overview of the current thyroid FNA practice in Taiwan, particularly data from our nationwide survey, describe the changes due to the new World Health Organization

(WHO) classification, and the advances of novel technologies.

CURRENT THYROID CYTOPATHOLOGY IN TAIWAN

In Taiwan, the majority of thyroid FNA cases are now performed with ultrasound guidance, since ultrasound-guided FNA provides better sampling of smaller or multiple nodules compared to palpation-guided FNA. High-resolution neck ultrasonography provides a simple, real-time, and noninvasive method to assess thyroid nodules. The accuracy of neck ultrasonography

in distinguishing malignant thyroid nodules was up to 86.8%, secondary to that of ultrasound-guided FNA (90.8%) [7]. The prevalence of thyroid abnormalities detected by ultrasonography in Taiwanese adults without palpable thyroid nodules was 18.5%, which were mostly cysts and small nodules [8]. Several published reports proposed sonographic criteria to prevent unnecessary ultrasound-guided FNA or further surgeries [9-11]. Analyzing data from Taiwan's National Health Insurance Research Database from 2004–2010, Lee et al. [12] reported that age-standardized rates of palpation-guided thyroid FNA and ultrasound-guided thyroid FNA increased by 10.9% and 349.3%, respectively. There was also a 94.8% increase in the age-standardized annual incidence rate of thyroid cancer. This is likely attributable to the widespread use of medical ultrasound for thyroid nodule evaluation. Despite the increasing trend of thyroid cancers, the mortality associated with thyroid cancer remains almost unchanged.

In regard to the cytology preparation and staining, the conventional smear for both Papanicolaou (on alcohol-fixed slides) and Liu's stain (on air-dried slides) are the most common methods for thyroid FNA cytology in Taiwan [6]. Although liquid-based cytology (LBC) is not currently prevalent, it has become increasingly popular due to easier specimen collection and transportation, standardized preparation, and reduction of various obscuring factors and artefacts [6,13]. Additionally, LBC provides specimens for further ancillary techniques, such as immunocytochemistry and molecular testing.

The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) is widely accepted for standardized terminology and better consistencies among cytopathologists and clinicians [14]. The six diagnostic categories are: unsatisfactory/nondiagnostic (US/ND), benign, atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), follicular neoplasm/suspicious for a follicular neoplasm (FN/SFN), suspicious for malignancy (SM), and malignant. Before TBSRTC, investigators in Taiwan used different diagnostic categories for thyroid FNA reporting [6,15].

In 2017, the Taiwan Society of Clinical Cytology performed the first nationwide survey to evaluate the general practice of thyroid cytopathology, application of the reporting system, and risk of malignancy (ROM). The questionnaires were sent to 119 cytology laboratories at medical centers, regional hospitals, and private clinical laboratories. Participants were asked seven questions as follows: (1) How many doctors are there signing out thyroid cytology reports?, (2) What are their subspecialties?, (3) How many cytotechnologists are there screening thy-

roid cytology?, (4) What were your annual thyroid cytology case numbers in 2015?, (5) What kind of reporting system is used in your laboratory for reporting thyroid cytology?, (6) Please provide your annual cases number, biopsy rate, and malignancy rate on follow-up for each diagnostic category, and (7) What are your cytology preparation methods for thyroid cytology in your laboratory? Fifty-five effective questionnaires (46%) were collected after a one-month answering period.

These responses from laboratories represented a total of 48,940 thyroid FNA cases annually in the year of 2015, and there were 143 pathologists, 28 endocrinologists, and 32 clinicians other than endocrinologists signing out thyroid cytology reports and 153 cytotechnologists screening thyroid cytology slides on a routine basis. As for the cytology preparation method, 78% of the laboratories used conventional smears, 18% used LBC, and 4% used concurrent conventional smears and LBC. For the reporting system, 64% of laboratories applied the traditional 4-tier system (negative, atypical, suspicious, and positive for malignancy), 31% adopted the TBSRTC, and 5% used other unspecified diagnostic systems (Table 1).

A total of 41,349 FNA cases could be converted to each corresponding TBSRTC diagnostic category. The rate of diagnosis, surgical resection, and malignancy for each category were as follows: US/ND: 24.04%, 1.96%, 15.9%; benign: 68.84%, 4.76%, 11.07; AUS/FLUS: 4.87%, 17.52%, 35.41%; FN/SFN: 0.35%, 44.06%, 49.21%; SM: 0.89%, 53.01%, 80.41%; malignant: 1.02%, 54.39%, 99.13% (Table 2). The nationwide survey demonstrated a high unsatisfactory rate (24.04%) and low rates

Table 1. Summary of the 2017 nationwide multicenter survey for thyroid cytopathology in Taiwan

	No. (%)
Institutes	55
Cases of thyroid FNA in 2015	48,940
Cytotechnologists	153
Professionals reporting thyroid cytology	
Pathologist	143
Endocrinologists	28
Clinicians other than endocrinologists	32
Institutions using different preparation methods	
Conventional smears	43 (78)
Liquid-based	10 (18)
Concurrent conventional and liquid-based	2 (4)
Institutions using different reporting systems	
TBSRTC	18 (33)
Traditional 4-tier system	34 (62)
Other unspecified systems	3 (5)

FNA, fine needle aspiration; TBSRTC, the Bethesda System for Reporting Thyroid Cytopathology.

Table 2. The rates of diagnosis, surgical resection, and malignancy for each diagnostic category of TBSRTC in Taiwan

Cytological diagnoses	FNA		Surgical resection		Histologically confirmed malignancy	
	No.	Proportion (%)	No.	Operation rate (%)	No.	ROM (%)
US/ND	9,940	24.04	195	1.96	31	15.90
Benign	28,464	68.84	1,355	4.76	150	11.07
AUS/FLUS	2,015	4.87	353	17.52	125	35.41
FN/SFN	143	0.35	63	44.06	31	49.21
SM	366	0.89	194	53.01	156	80.41
Malignant	421	1.02	229	54.39	227	99.13
Total	41,349	100.00	2,389	5.78	720	30.14

TBSRTC, the Bethesda System for Reporting Thyroid Cytopathology; FNA, fine needle aspiration; ROM, risk of malignancy; US/ND, unsatisfactory/nondiagnostic; AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; SM, suspicious for malignancy.

of indeterminate diagnoses (AUS/FLUS, 4.87%; FN/SFN, 0.35%) for thyroid FNA cytology in Taiwan. The high unsatisfactory rate might be attributable to the routine submission of the fluid from symptomatic hemorrhagic cysts for cytologic examination in our clinical practice. Therefore, a different diagnostic approach for the specimen with cystic fluid only should be considered, such as in the Japanese system, to reduce the unsatisfactory rate. The low rates of indeterminate diagnoses reflected the application of relatively strict criteria for using these categories to avoid clinical uncertainty in the overall management. Moreover, compared to the Western experience [16], the ROM in benign, AUS/FLUS, and FN/SFN in this survey was higher and the resection rate for these nodules were lower. These results suggested a more conservative approach and the application of other clinicoradiological parameters to select eligible patients for surgeries in our clinical practice. Nevertheless, the general characteristics of the thyroid FNA in Taiwan, including fewer malignant FNAs and lower ROMs of all resected nodules, were also different from other Asian countries [16] and deviated more towards the results for Western countries. This implies that the guidelines from Western countries were more closely followed by our clinicians. The major limitation of the present survey was that the data was derived from the questionnaire and there might be some bias regarding the evaluation of the incidence and malignancy rate.

IMPACT OF NONINVASIVE FOLLICULAR THYROID NEOPLASM WITH PAPILLARY-LIKE NUCLEAR FEATURES

In 2016, the new terminology of noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) was proposed to replace the noninvasive encapsulated follicular variant of papillary thyroid carcinoma (PTC) [17] and this has

been further adopted by the 2017 World Health Organization Classification of Tumors of Endocrine Organs [18]. The change in tumor classification inevitably influences the preoperative thyroid FNA diagnosis [19] and the utilization of molecular testing [20]. However, an international multi-institutional study from India, Japan, South Korea, Taiwan, and Thailand revealed a less significant impact of NIFTP reclassification on the practice of thyroid cytopathology [21]. In Taiwan, NIFTP cases constituted only 2.9% of excised thyroid nodules and 5.3% of all malignancies, and most NIFTP cases (66.1%) were interpreted as indeterminate FNA categories (FN/SFN, 32.2%; AUS/FLUS, 22.0%). The differences in the ROM before and after NIFTP reclassification were not statistically significant for all the diagnostic categories.

IMMUNOCYTOCHEMISTRY, MOLECULAR TESTING, AND COMPUTERIZED CYTOMORPHOMETRY

In thyroid FNA, immunocytochemistry had been implemented to differentiate nonfollicular lesions from thyroid follicular lesions [22–24]. Immunocytochemistry may improve the cytologic diagnostic accuracy, but does not have a consistent predictive value for malignancy [24]. For immunocytochemistry, a cell block is usually preferable compared with cytologic specimens, however, thyroid FNA materials might be limited for cell block preparation.

The first report on molecular testing in thyroid FNA from Taiwan can be traced back to 2003. Liou et al. [25] found that human telomerase reverse transcriptase (*TERT*) gene expression was more prevalent in malignant thyroid FNA samples than in the benign thyroid FNA samples and proposed that it is an adjunctive molecular marker for the preoperative diagnosis of thyroid malignancies. The need for distinguishing benign thyroid

lesions from thyroid cancers in thyroid FNA has led to the investigation of differentiating molecular markers. To date, more sophisticated molecular tests for thyroid FNA are commercially available, such as the ThyroSeq, Afirma, RosettaGX Reveal, ThyGenX, and ThyraMIR [26]. These molecular tests have high negative predictive values that range from 92% to 97% and lower positive predictive values that range from 37% to 83% for indeterminate thyroid nodules; therefore, these are more ideal as “rule out” tests. However, these tests are rarely applied in Taiwan due to the high costs.

The application of computerized cytomorphometry had been used as an emerging alternative test to stratify risks in thyroid nodules with indeterminate FNA results in Taiwan [27,28]. Computerized cytomorphometry, serving as a sequential reader, analyzes objective quantification of selected morphologic and chromatic parameters in individual cells on cytology slides [28]. For example, using computerized cytomorphometry, the nucleus-cell ratio and variation of the nuclear area showed significantly positive correlations with PTC recurrence and could be predictors of recurrence [28]. With 100% sensitivity, the computerized quantification of cytological characteristics could assist in differentiating 17.6% of AUS/FLUS, 13.6% of FN/SFN, and 33.3% of SM cases as benign rather than malignant, avoiding unnecessary thyroidectomy [29]. The cytological features used in this computerized method included the mean nuclear size, mean nuclear elongation, nuclear-to-cytoplasmic saturation ratio, nuclear-to-cytoplasmic ratio, nuclear polarity, and inclusion index. A larger nuclear size and higher nuclear-to-cytoplasmic ratio were related to malignancy. This computerized method was developed to assist cytopathologists and clinicians in cases of indeterminate thyroid cytology. The limitation of this technology is that it was developed solely for evaluating nuclear features of PTC and was not useful for differentiating other follicular-patterned neoplasms, particularly borderline tumors such as NIF-TP and well-differentiated tumor/follicular tumors of uncertain malignant potential.

FINE-NEEDLE ASPIRATION CYTOLOGY AND INTRA-OPERATIVE FROZEN SECTIONS FOR THYROID SURGERY

Traditionally, intra-operative frozen section was largely applied to confirm the cytological interpretation and to identify malignancy in patients with indeterminate or unsatisfactory cytological diagnoses in Taiwan [1]. Currently, most endocrine surgeons still prefer frozen sections as an intra-operative guide for thyroid

surgery to reduce unnecessary extensive surgery or the chance of repeated operations. Intra-operative frozen sections provide valuable information, especially in rare cancers [29,30].

However, it is extremely difficult to diagnose follicular thyroid carcinoma (FTC) either by using FNA or intra-operative frozen section. In a retrospective study of 22,134 FNA cases, only 23 cases (46%) were intra-operatively diagnosed as malignant by frozen section among 50 cases of FTC, and 13 cases (26%) were intra-operatively diagnosed as benign nodules (nodule hyperplasia and adenoma) [31]. Similarly, Hürthle cell carcinoma (HTC) is difficult to diagnose by intra-operative frozen section. Lee et al. [32] reported that 60% of surgical cases with HTC were designated as malignancies based on frozen sections. A meta-analysis for Hürthle cell lesions in thyroid FNA in nine institutions from six Asian countries revealed that there was interinstitutional variation in the cytologic interpretation of Hürthle cell morphology and in Bethesda categorization [33]. Hürthle cell-rich aspirates were most frequently categorized as AUS/FLUS, followed by FN/SFN. Only 13% of Hürthle cell-rich lesions were actually malignant. Clinical risk factors, such as age less than 20 years and history of autoimmune thyroid disease might be parameters for selecting patients for observation or surgery, since the frozen section approach was valuable in deciding the extent of thyroidectomy in patients with follicular and Hürthle cell neoplasms [32].

CONCLUSION

Thyroid FNA cytology is a cost-effective and reliable method for evaluating thyroid nodules in Taiwan, and is typically performed under ultrasound guidance. The sonographic pattern plays a major role and is the deciding factor for aspiration. Even with the recent coronavirus disease 2019 (COVID-19) pandemic, the procedures and preparations are performed according to national and institutional laboratory biosafety guidance [34,35]. Although most laboratories performed conventional smears, 22% of institutions had applied LBC in thyroid cytology. For the reporting system, 64% of laboratories followed the traditional 4-tier system, and 31% had adopted the TBSRTC. In general, the cytopathology laboratories in Taiwan are in transition and plan to gradually accept TBSRTC to allow easy and reliable data sharing for national and international collaboration and comparison. Newly-developed thyroid cytology technologies, such as immunocytochemistry, molecular tests, and computerized cytomorphometry, may further facilitate cytology diagnoses. Finally, intra-operative frozen consultation may serve as a complementary test, except for patients with follicular and Hürthle

cell neoplasms.

Ethics Statement

Not applicable.

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Conflicts of Interest

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

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Pathologic interpretation of endoscopic ultrasound–guided fine needle aspiration cytology/biopsy for pancreatic lesions

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Pathologic interpretation of endoscopic ultrasound–guided fine needle aspiration (EUS-FNA) cytology/biopsy specimens is one of the most challenging tasks in cytology and surgical pathology practice, as the procedure often yields minimal amounts of diagnostic material and contains contaminants, such as blood cells and normal intestinal mucosa. EUS-FNA cytology/biopsy will nevertheless become a more popular procedure for evaluation of various pancreatic lesions because they are difficult to approach with conventional endoscopic procedures. Pathologists should understand the structural differences and limitations of EUS-FNA that make pathologic diagnosis difficult. Ancillary tests are available for differential diagnosis of EUS-FNA for various pancreatic lesions. Immunostains are the most commonly used ancillary tests, and pathologists should be able to choose the necessary panel for differential diagnosis. Pathologists should review clinical history and radiologic and/or EUS findings before selecting an immunostain panel and making a pathologic diagnosis. In addition, one's threshold of malignancy should be adjusted according to the appropriate clinical setting to avoid under-evaluation of pathologic diagnoses. Clinico-pathologic correlation is essential in pathologic evaluation of EUS-FNA for pancreatic lesions. Pathologists can reduce errors by correlating clinical and radiologic findings when evaluating EUS-FNA. Some molecular tests can be applied in differential diagnosis of pancreatic neoplastic and cystic lesions. Molecular data should be used as supportive evidence of a specific disease entity, rather than direct evidence, and should be correlated with clinico-pathologic findings to avoid errors in pathologic diagnosis.

Key Words: Endoscopic ultrasound-guided fine needle aspiration; Pancreatic neoplasms; Pathology

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Endoscopic ultrasound–guided fine needle aspiration (EUS-FNA) cytology/biopsy is currently performed at many institutions and has become a routine procedure for pathologic diagnosis of pancreatic lesions. Although endoscopic ultrasound scanning began in the early 1980s [1], it has gained widespread popularity since the introduction of fine needle aspiration cytology/biopsy devices, which allow guided biopsies of target lesions visualized by endoscopic ultrasound [2]. Before the era of EUS-FNA, it was difficult to target pancreatic lesions due to limited accessibility by percutaneous needle biopsies. Hence, pathologists could rarely encounter cytology and/or biopsy material from pancreatic lesions. With the increasing popularity of EUS-FNA procedures, interpretation of EUS-FNA cytology/biopsy material has become an almost inevitable part of routine practice for pathologic diagnosis of various pancreatic lesions. A main con-

cern of pancreatic EUS-FNA specimen interpretation is the limited amount of aspirated material. Compared to aspirated material from superficial organs, such as the thyroid, breast, or uterine cervix, EUS-FNA from pancreatic lesions provides aspirates with relatively limited cellularity that may seem inadequate to less experienced pathologists. However, EUS-FNA inevitably results in lower cellularity compared to aspirates from other superficial organs. To make an appropriate diagnosis, pathologists should consider the clinical impression, especially radiologic and/or EUS findings; otherwise, many EUS-FNA specimens may be interpreted as “inadequate specimen”. It is occasionally necessary to deem the sample “inadequate”, “atypical”, or of “uncertain malignant potential”, which are diagnostically less useful to clinicians, but necessary. The use of these indeterminate categories will be reduced with expertise and correlation of cytologic

findings with the clinico-radiological findings. If these categories are overused, communication and trust between clinicians and pathologists may become compromised. Pathologists may require time and experience to adjust to the relatively paucicellular smears of EUS-FNA specimens and their potential artifacts, including contaminants. In this review, we summarize the key cytologic findings of common pancreatic lesions, review pre-analytic parameters that may affect the pathologic diagnosis of EUS-FNA, and tips for clinicopathologic correlation that may be helpful in the differential diagnosis of EUS-FNA for various pancreatic lesions.

DIAGNOSTIC CONSIDERATIONS

Different preparation methods

In most institutions, pancreatic EUS-FNA aspirates are prepared for cytologic review by the direct smear method. Direct smears are usually performed in the endoscopy suite; as soon as the aspirated material is obtained, the material is expelled onto glass slides. Grossly visible solid particles are placed into formalin for histopathologic examination, while the remaining material is smeared on site. The smears are either placed directly into alcohol fixative for Papanicolaou or hematoxylin-eosin stain or air-dried for subsequent Diff-Quik stain. If smeared properly without crushing or air dry artifacts, Papanicolaou or hematoxylin-eosin stains result in smears with high nuclear detail, which is important for pathologic diagnosis of various pancreatic neoplasms. Direct feedback helps to improve cytology smears, including microscopic observation of the smear slide by the person who actually performs the smear. Unfortunately, cytology

smear preparation and cytology interpretation are usually performed by different people. Romanowsky stains, such as Diff-Quik, are useful for analyzing background material, such as mucin, and for examining cytoplasmic features. Cell blocks, which may be useful for ancillary immunohistochemical stains, may also be prepared from aspirates. Recently, liquid-based cytology (LBC; e.g. ThinPrep, SurePath) has also been used for pancreatic EUS-FNA cytology.

Liquid-based cytology

LBC has become a standard method for cytologic evaluation of some organs, including the uterine cervix and thyroid. LBC can eliminate unnecessary background inflammatory and blood cells to increase diagnostic accuracy. Recent studies reported that LBC showed similar results to conventional smear for diagnosis of pancreatic masses [3,4]. The use of LBC may be limited for mucinous neoplasms because mucin is more dilute and scant on LBC preparations.

Contaminants

Because EUS-FNA involves penetration of either the gastric or duodenal wall to access the pancreas, most EUS-FNA aspirates contain at least a small amount of normal gastrointestinal mucosa. This is a very important pitfall in the interpretation of pancreatic EUS-FNA specimens, as normal gastrointestinal contaminants may be mistaken for neoplastic epithelial cells. Gastric foveolar epithelium appears as large irregular folded or monolayered sheets of mucin-containing columnar cells (Fig. 1A). The columnar cells have basally oriented nuclei with a palisaded appearance, and the luminal border is frequently seen

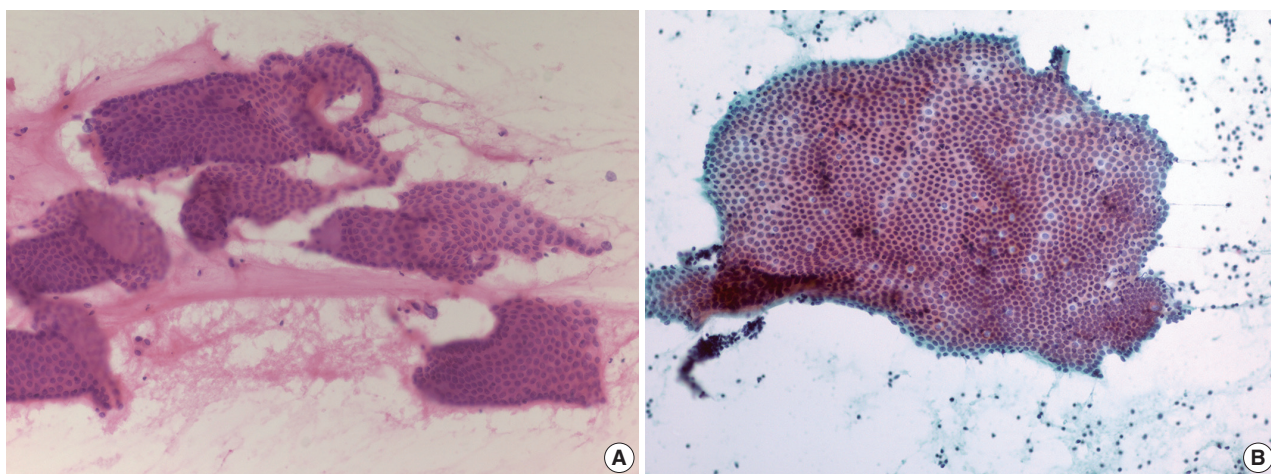


Fig. 1. (A). Normal gastric mucosa in conventional cytology smear shows nests of monotonous cells with smooth boundaries. (B) Normal duodenal mucosa showing scattered goblet cells in conventional cytology smear (Pap smear).

along one edge of the epithelial cell sheets. Duodenal epithelium is also characterized by sheets of columnar cells with interspersed goblet cells, resulting in a characteristic “starry-sky” appearance (Fig. 1B). However, the most common contaminants are red blood cells, which obscure the cytologic feature of EUS-FNA aspirates. Cell blocks are alternative tools to help overcome bloody smears; however, the efficiency of conventional cell block preparation is sometimes suboptimal. The tissue coagulum clot method reportedly increases cell block cellularity with endobronchial ultrasound-guided transbronchial fine-needle aspiration [5]. However, more validation studies are needed to apply this method to EUS-FNA for pancreatic lesions.

Interpretation of pancreatic cystic lesions

As cystic lesions comprise 2%–13% of pancreatic lesions [6], it is not uncommon for pathologists to encounter EUS-FNA specimens for pancreatic cystic lesions. While characteristic cytologic features may help in diagnosis when a mural or papillary nodule is present within the pancreatic cystic lesion, purely cystic lesions without mural or papillary nodules may yield paucicellular smears or only scattered macrophages. These cases would be interpreted as “inadequate” according to ordinary criteria for cytology diagnosis. However, it is difficult to obtain adequate cellularity from these cystic lesions despite repeated procedures. This is especially true for unilocular cystic lesions, such as pseudocysts, macrocystic serous or mucinous cystadenomas, and cystically dilated intraductal papillary mucinous neoplasms (IPMNs) without mural nodules; if the gross and microscopic findings of these cystic lesions are considered, it is logical that not enough cells or tissue may be obtained by the EUS-FNA procedure. In this situation, it may be more reasonable to interpret the findings as “benign cystic lesion” than “inadequate specimen.” Although a hidden malignancy may exist around the “benign cystic lesion”, this pattern is very rarely identified. In daily practice, benign cystic lesions, such as serous or mucinous cystadenomas, cystic IPMNs, and pseudocysts, are more common. Pathologists should correlate radiologic and EUS findings to avoid under-evaluation of pathologic diagnosis for these cystic lesions. If imaging and EUS findings are concordant, it is not difficult to predict the biologic behavior of pancreatic cystic lesions. However, discrepancy between imaging and EUS interpretation for these cystic lesions is not uncommon. In this scenario, pathologists should raise the threshold for malignancy in pathologic diagnosis of EUS-FNA. Clinico-pathologic correlation is important for adequate pathologic interpretation of EUS-FNA from various pancreatic lesions.

KEY PATHOLOGIC FEATURES OF COMMONLY ENCOUNTERED PANCREATIC NEOPLASMS

Ductal adenocarcinoma

Pancreatic ductal adenocarcinoma is the most common solid tumor of the pancreas and the most commonly encountered pancreatic neoplasm on EUS-FNA. Aspirated material usually demonstrates atypical cells with or without a necrotic background. The cellularity of the cytologic smear depends on many parameters, including tumor size, location, cellularity, type of aspiration needle, and endoscopist technique [7–9]. A pathologist is more likely to encounter a paucicellular smear than a highly cellular smear. If many atypical cells are found in a necrotic background (Fig. 2A), it is easy to conclusively diagnose a malignancy even without clinical information. However, pathologists may encounter paucicellular smears showing only a few atypical cells without a necrotic background. In such situations, it is difficult to differentiate reactive ductal cell atypia of chronic pancreatitis from malignancy. Pathologists should correlate with the clinical impression and radiologic findings of these pancreatic lesions. If the clinical finding impression is an overt malignancy, such as pancreatic tumor with vascular encasement or liver metastasis, the pathologic diagnosis of malignancy can be made with a very limited volume of atypical cells. Pancreatic ductal adenocarcinomas are often well-differentiated with very mild cytological atypia, and one should be careful not to mistake them for contaminated gastric foveolar epithelium. In such cases, the architecture of cell groups should be observed in detail, including cribriforming, nuclear stratification, and loss of regular honeycombing seen in normal and benign epithelia (“drunken honeycomb”). Well differentiated tumor cell nests usually exhibit more cellularity with overlapping and touching nuclei (Fig. 2B). The presence of scattered small clusters of atypical cells with necrotic cell debris in the background is also helpful, as pancreatic ductal adenocarcinomas frequently demonstrate necrotic backgrounds and scattered small atypical cell nests or clusters. However, this necrotic background should be discriminated from the fibrin clots that are frequently observed in bloody smears (Fig. 2C). Another EUS-FNA specimen that demonstrates diffuse necrosis is tuberculous lymphadenitis involving peripancreatic or retroperitoneal lymph nodes: if the EUS-FNA cytology/biopsy shows a diffusely necrotic background without apparently viable tumor cells (Fig. 2D), the pathologist should check the clinical impression and imaging findings. In many cases, the radiologic impression is lymphoma, especially if there are multiple enlarged lymph nodes around the

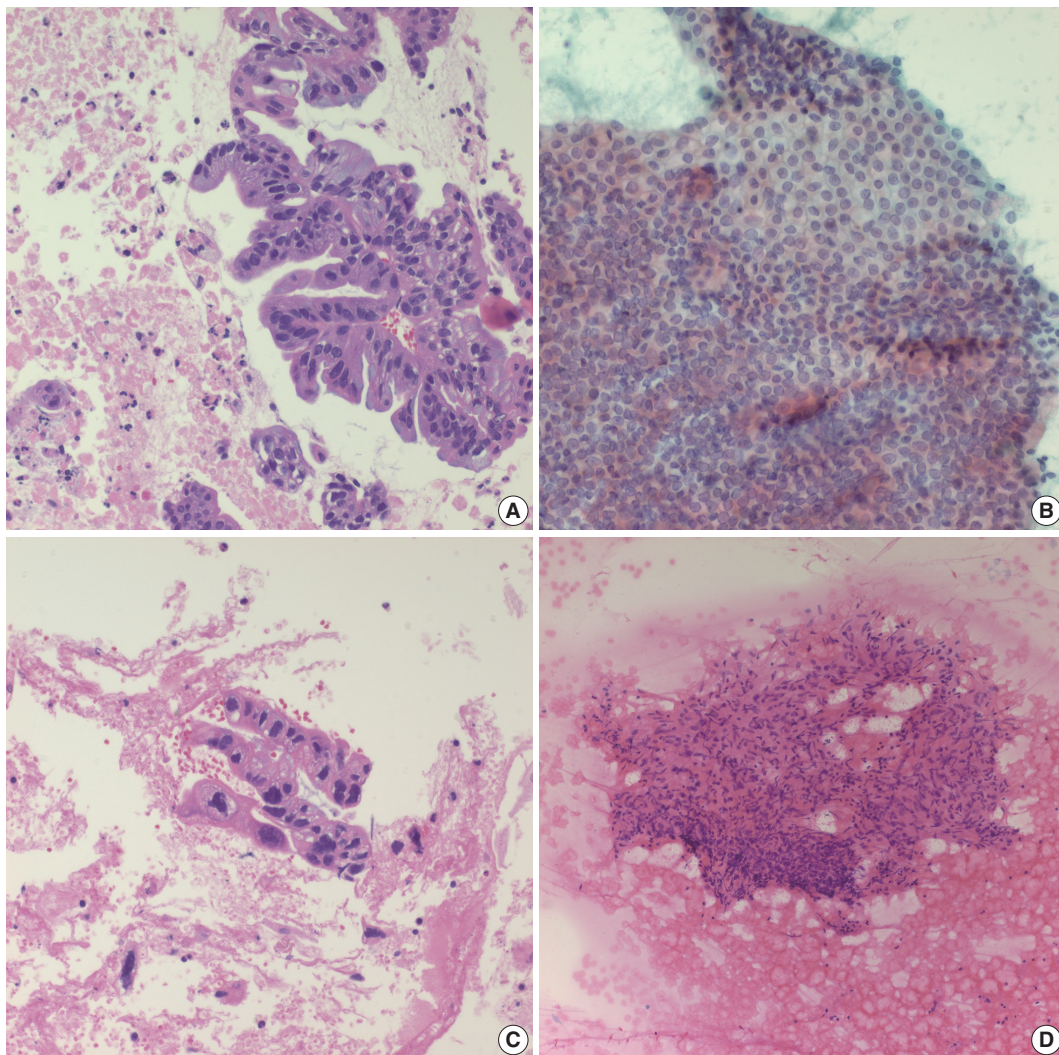


Fig. 2. (A) Ductal adenocarcinoma showing scattered atypical cell nests with complex papillary architecture and necrotic background on biopsy slide. (B) Well differentiated ductal adenocarcinoma cell nest, mimicking normal mucosa, is more hypercellular on conventional cytology smear (Pap). (C) A few atypical tumor cells in a fibrinous background on biopsy slide. Although it does not include necrotic tumor cell debris, the cytologic atypia is sufficient for a diagnosis of malignancy, especially when clinical or radiologic evaluation strongly suggests a malignancy. (D) Tuberculous inflammation shows aggregates of granuloma without viable tumor cells in conventional cytology smear.

pancreas or retroperitoneal region. Due to the high prevalence of tuberculosis in Korea, the possibility of tuberculous lymphadenitis should be considered, especially in EUS-FNAs from older patients. Special stains for acid-fast bacilli and molecular studies can be performed with biopsies or cell blocks to confirm the diagnosis of tuberculosis. Diffuse necrotic backgrounds are not limited to ductal carcinoma or tuberculosis. Necrotic cells may also be seen in EUS-FNA cytology/biopsy of solid pseudopapillary neoplasms (SPNs) and poorly differentiated neuroendocrine carcinomas. However, SPN and neuroendocrine carcinoma are usually accompanied by hypercellular components on cytologic smears, and these neoplasms are relatively easily differentiated

on radiologic evaluation. The main differential diagnosis points are summarized in Table 1.

Intraductal papillary mucinous neoplasm

IPMN is one of the most common pancreatic cystic neoplasms, and EUS-FNA is frequently performed to evaluate the grade of cytologic atypia and presence of an associated invasive carcinoma. It is not uncommon to find mucinous content in cytologic smears from EUS-FNA. Although mucin may be found in EUS-FNA material from IPMN, it is not a specific finding. When a mucinous background is noted on cytology/biopsy slides (Fig. 3A), the pathologist should check the radiology findings. If the

radiologic interpretation strongly suggests IPMN, the pathologist may suggest IPMN in the cytopathologic diagnosis of EUS-FNA. However, diagnosis of IPMN on EUS-FNA should be avoided if the radiologic and EUS findings do not suggest IPMN, as the radiologic findings of IPMN are relatively unique. In addition, it is relatively uncommon to observe mucin in cytology slides and/or biopsies from IPMN, especially in LBC. The appearance of aspirated mucin also depends on its vis-

Table 1. Differential diagnosis in EUS-FNA of common pancreatic neoplasms

	DAC	IPMN	NET	SPN
Cytologic atypia	+, ++, +++	+	+	+, ++ ^a
Discohesive pattern	+ ^b	-	++, +++ ^c	++, +++ ^d
Organoid cell nest	+, ++	+	-	-
Necrotic background	+, ++, +++ ^e	-	-	+ ^f
Mucin	+ ^g	+, ++ ^h	-	-

EUS-FNA, endoscopic ultrasound-guided fine needle aspiration; DAC, ductal adenocarcinoma; IPMN, intraductal papillary-mucinous neoplasm; NET, neuroendocrine tumor; SPN, solid pseudopapillary neoplasm.

^aSome cases of SPN may show scattered large bizarre nuclei, which correspond to degenerative atypia and do not indicate high-grade malignancy; ^bDiscohesive atypical cells may be present in the background of tumor necrosis; ^cNET usually has a hypercellular smear with diffuse discohesive pattern and mild cytologic atypia; ^dSPN frequently exhibits plasmacytoid features and occasional clear cytoplasm; ^eDAC exhibits a necrotic background composed of necrotic tumor cells and inflammatory cells; ^fSPN may show necrotic cells that are mostly hemorrhagic, which is different from tumor necrosis of DAC; ^gA mucinous component may be present in EUS-FNA cytology/biopsy from DAC. However, it is frequently mixed with a necrotic background and atypical tumor cells; ^hIPMN may have a diffuse mucinous background. However, it depends upon the viscosity of mucin.

cosity. Main duct type IPMNs with intestinal phenotype usually show viscous mucin, similar to that seen in appendiceal mucinous neoplasms. Conversely, branch duct type IPMNs contain relatively less viscous gastric foveolar-type mucin, resulting in a more transparent mucinous background that is difficult to recognize on cytologic smears. The typical cytologic features of IPMNs include papillary clusters of columnar epithelial cells containing cytoplasmic mucin (Fig. 3B), and IPMNs may be graded according to the degree of nuclear atypia. IPMNs without intraductal or intracystic papillary lesions may not demonstrate typical papillary epithelial cell nests on EUS-FNA slides. Sometimes it is difficult to differentiate papillary cell nests from contaminated intestinal mucosa. If the IPMN is branch duct type with gastric foveolar type epithelium, it may be difficult to differentiate between mucinous epithelium of the IPMN and contaminated gastric mucosa. Pathologists should assess the radiologic findings of IPMN. If there are no mural or papillary nodules of IPMN on radiologic evaluation, the cellular nest of epithelial mucosa probably corresponds to contaminated gastric mucosa, not the epithelial component of IPMN. The presence of scattered goblet cells within monotonous-looking cell nests is a helpful clue that can differentiate normal intestinal mucosa from IPMNs (Fig. 1B). It is therefore important that pathologists assess the anatomic location of target lesions on clinical EUS-FNA reports; lesions in the pancreatic head and uncinate process are usually targeted via the duodenal wall, while those

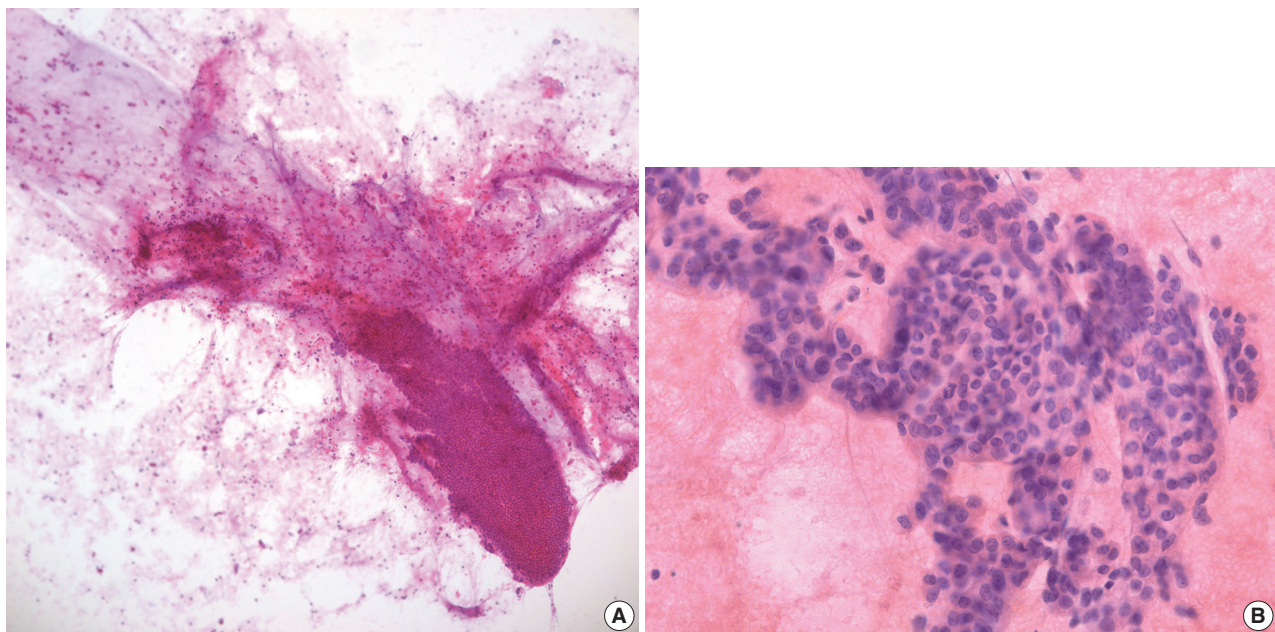


Fig. 3. Intraductal papillary mucinous neoplasm. (A) A mucinous background is seen on conventional cytology smear. (B) A papillary epithelial nest showing high-grade dysplasia on conventional cytology smear.

located in the body and tail are usually accessed via the gastric wall. If pathologists identify small intestinal mucosa-like epithelial cell nests in EUS-FNA from the pancreas body or tail with a clinical impression of main duct type IPMN, it likely corresponds to the epithelial component of IPMN because the EUS-FNA needle penetrated the gastric wall, not the duodenal mucosa. However, if radiologic findings indicate branch duct type IPMN, which is mostly composed of gastric phenotype epithelium, it may be difficult to discriminate between contaminated gastric mucosa and the epithelial component of gastric phenotype IPMN. The papillary nests within cystic lesions are relatively small in branch duct type IPMN. If a pathologist finds relatively large nests of intestinal mucosa, it may correspond to contaminant intestinal mucosa rather than papillary nests of branch duct type IPMN.

Serous/mucinous cystic neoplasm

Serous cystic neoplasms (SCNs) are not infrequently submitted for EUS-FNA evaluation. It is uncommon to observe the typical serous lining cells on cytology smears and/or biopsy, especially in aspirates from unilocular or macrocystic lesions. However, if the SCN is a multilocular cystic lesion, some serous epithelial cell clusters composed of cuboidal cells with clear cytoplasm may be seen in cytology and/or biopsy slides. If the pathologist can find scattered subepithelial capillaries in the biopsy slide, it is supportive of an SCN. In most cases, SCN is easily detected by radiologic evaluation, except for macrocystic SCN. Radiologic correlation is essential in pathologic diagnosis of SCN in EUS-FNA.

Mucinous cystic neoplasm (MCN) is a relatively uncommon cystic neoplasm of the pancreas. Mucinous epithelial lining and an underlying ovarian-type stroma are characteristic histologic findings. However, it may be difficult to observe well-preserved mucinous epithelium and ovarian-type stroma, even in resected specimens. It is even harder to identify mucinous epithelial cells and/or ovarian-type stroma in EUS-FNA cytology/biopsy. If some mucinous epithelial cells are seen, the pathologist should check the clinical findings and radiologic interpretation. The characteristic clinical setting is a middle-aged female with a pancreatic unilocular cystic neoplasm or multilocular septated cyst without ductal communication. Invasive carcinomas may arise from MCNs, and the biologic behavior of MCN-associated invasive carcinomas is aggressive even at an early stage. It may be difficult to detect early invasion by EUS-FNA, as these early invasive components do not create mass lesions on radiologic or EUS findings.

Neuroendocrine neoplasms

Aspirated material from pancreatic neuroendocrine neoplasms usually results in a hypercellular smear. Although some high-grade neuroendocrine carcinomas have an accompanying necrotic background, well-differentiated pancreatic neuroendocrine tumors (NETs) rarely show necrosis on cytology smears. Pancreatic NET is classified by a 3-tier scheme [10]. Grade 1 and 2 NETs are well-differentiated neuroendocrine tumors with mitotic rate of $< 2/2 \text{ mm}^2$ or Ki-67 index $< 3\%$ and mitotic rate of $2\text{--}20/2 \text{ mm}^2$ or Ki-67 index $3\%\text{--}20\%$, respectively. Well-differentiated grade 3 NETs show a mitotic rate of $> 20/2 \text{ mm}^2$ or Ki-67 index $> 20\%$. Tumor cells from well-differentiated NETs usually show mild cytologic atypia, sometimes with characteristic salt-and-pepper chromatin pattern, which is more frequently found on cytology (Fig. 4A). NET cells on biopsy slides are more discohesive epithelial cells with round to vesicular nuclei and plump cytoplasm (Fig. 4B). While NETs usually demonstrate scant cytoplasm, some NETs may have relatively abundant cytoplasm (Fig. 4C). In such cases, it may be difficult to distinguish NET from SPN. The cytologic findings of discohesive cells and plasmacytoid features suggest the diagnosis of SPN. Immunostaining for β -catenin is a useful and reliable differential diagnostic tool, as other neuroendocrine markers (e.g., CD56, synaptophysin) can be expressed in both tumors [11]. NETs usually express β -catenin with a membranous pattern whereas SPNs exhibit nuclear β -catenin staining. Pathologists should correlate with radiologic findings before performing immunohistochemical stains. NETs are usually well demarcated hyper-enhancing masses. If a pancreatic mass is partially solid and cystic on radiologic evaluation, it is more likely to be an SPN than a NET. Grading of NET on EUS-FNA biopsies is well correlated with that of surgically resected tumors [12,13]. However, considering the uneven distribution of Ki-67 hot spots in tissue sections, it is still possible that the Ki-67 labeling index of a biopsy does not represent the proliferation index of the whole tumor [14]. Aspirates from high-grade small cell neuroendocrine carcinomas demonstrate hyperchromatic small cell carcinoma-like features with or without a necrotic background (Fig. 4D). Differential diagnosis of metastatic lung small cell from may be difficult because the cytologic findings are similar in both lesions. Ancillary immunostaining for thyroid transcription factor 1 (TTF-1) may be helpful, as metastatic small cell carcinoma from the lung usually expresses TTF-1 immunoreactivity. However, pathologists should also correlate with the clinical history and radiologic findings for systemic metastasis. If patients have a past medical history of lung cancer with multiple metastasis,

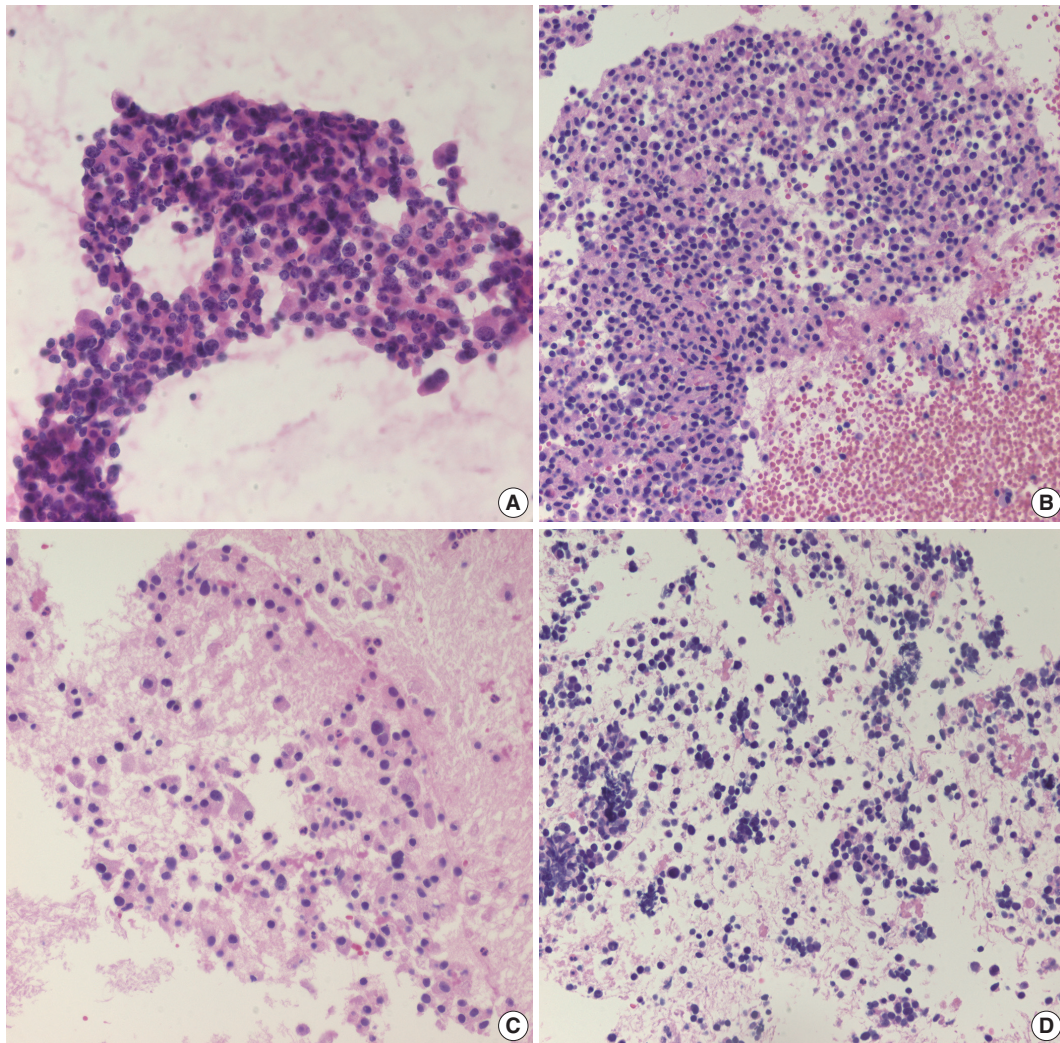


Fig. 4. (A) Well-differentiated neuroendocrine tumor cells show the characteristic salt-and-pepper chromatin pattern on conventional cytology smear. (B) On biopsy, well-differentiated neuroendocrine tumor cells show diffuse nest-like arrangement of monotonous nuclei with mild atypia. (C) Scattered epithelioid cells with hyperchromatic nuclei and abundant cytoplasm may be seen in well-differentiated neuroendocrine tumors. (D) Poorly differentiated neuroendocrine carcinoma shows scattered hyperchromatic nuclei.

it should be easily suspected regardless of ancillary test results.

Solid pseudopapillary neoplasm

The characteristic clinical setting of SPN is a middle age female with a pancreatic mass in a distal pancreas location, either the body or tail. Although exceptional cases may occur, pathologists should assess these two clinical findings when diagnosing SPN. Radiologic examination typically shows a solid and cystic tumor with or without necrotic contents. However, SPNs smaller than 2 cm may present as solid tumors and may be interpreted as NETs on radiologic evaluation. Cytologic smears of SPN are usually discohesive and hypercellular, and the cells demonstrate mild nuclear atypia and plasma cell-like features (Fig. 5A).

The presence of myxoid pseudopapillae is a characteristic pathologic finding of SPN. However, it is relatively uncommon to see the typical myxoid pseudopapillary pattern of SPN in cytologic smears of EUS-FNA (Fig. 5B). Scattered pleomorphic or bizarre cells from SPN are reported to reflect degenerative change rather than high-grade malignancy, which may be overestimated on EUS-FNA pathologic diagnosis [15]. Sometimes, tumor cells of SPN show clear cell change that mimics renal cell carcinoma. Pathologists should correlate with the patient's past medical history before confirming a diagnosis of metastatic renal cell carcinoma to the pancreas.

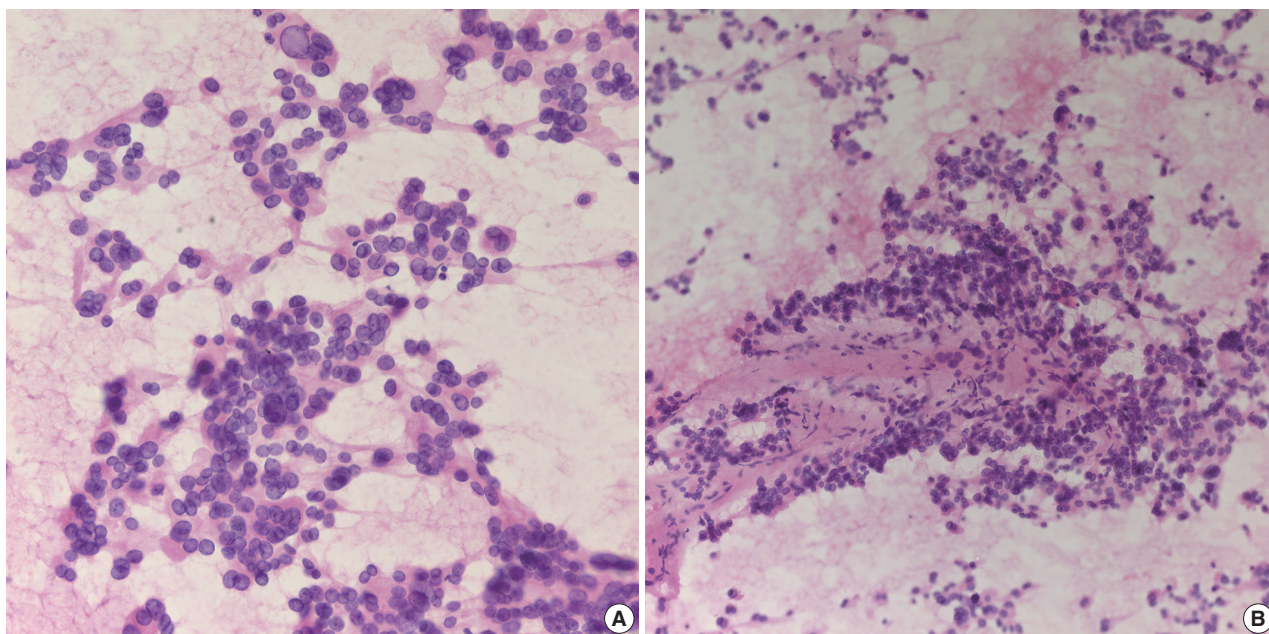


Fig. 5. (A) Scattered solid pseudopapillary neoplasm cells show discohesive organoid pattern with vesicular nuclei. (B) Solid pseudopapillary neoplasms show myxoid pseudopapillae with scattered plasmacytoid cells.

Autoimmune disease

EUS-FNA is rarely performed to evaluate autoimmune disease that involves the pancreas or bile duct. If the clinical history or radiologic findings are characteristic, the pathologic findings of EUS-FNA biopsy may provide typical histologic findings of dense lymphoplasmacytic infiltration and/or storiform fibrosis. However, if the patient history or radiologic findings are vague, it may be difficult to differentiate conventional chronic pancreatitis from autoimmune pancreatitis or cholangitis. Especially when presenting as a local mass-forming lesion, it is very difficult to suspect autoimmune disease if EUS-FNA cytology/biopsy provides only surface mucosa [16]. It should be emphasized that the pathologic diagnosis of autoimmune disease should be made after excluding malignancy in the differential diagnosis. IgG4 immunostaining is essential in evaluation of IgG4-related autoimmune disease that involves the pancreas or bile duct. If IgG4 positivity is sufficient by immunostain study, IgG4-related autoimmune disease can be diagnosed. However, pathologists should be careful to exclude autoimmune disease with insufficient IgG4 positivity because increased IgG4-positive plasma cells can be found in other inflammatory conditions or even in some malignancies [17].

Metastatic tumor

Some malignant tumors may metastasize to the pancreas; renal cell carcinoma is one of the most common metastatic tumors in the pancreas. However, it is uncommon for pathologists

to see metastatic tumors on EUS-FNA cytology and/or biopsy because it is difficult to evaluate metastasis on pancreas EUS-FNA. It is challenging to consider metastatic renal cell carcinoma without pertinent clinical information. Metastatic renal cell carcinoma sometimes does not exhibit characteristic clear cell features on EUS-FNA cytology and/or biopsy specimens, instead showing monotonous cell nests with abundant capillaries (Fig. 6A). Metastatic small cell carcinoma from the lung is difficult to differentiate from primary poorly differentiated neuroendocrine carcinoma of the pancreas because the pathologic findings are very similar (Fig. 6B). Immunostain for TTF-1 and correlation with clinical history are essential for differential diagnosis of metastasis.

WHAT ANCILLARY TESTS CAN BE APPLIED TO ENDOSCOPIC ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION?

Many ancillary tests can be applied for pathologic evaluation of EUS-FNA specimens. Immunohistochemistry on biopsy specimens or cell blocks is the most widely used method. Immunohistochemical stains are particularly helpful in the differential diagnosis of NETs (e.g., CD56, synaptophysin, chromogranin), SPNs (e.g., nuclear β -catenin, CD10), acinar cell neoplasms (e.g., trypsin, chymotrypsin, BCL10), and various metastatic tumors [11,18,19]. In addition, Ki-67 labeling indi-

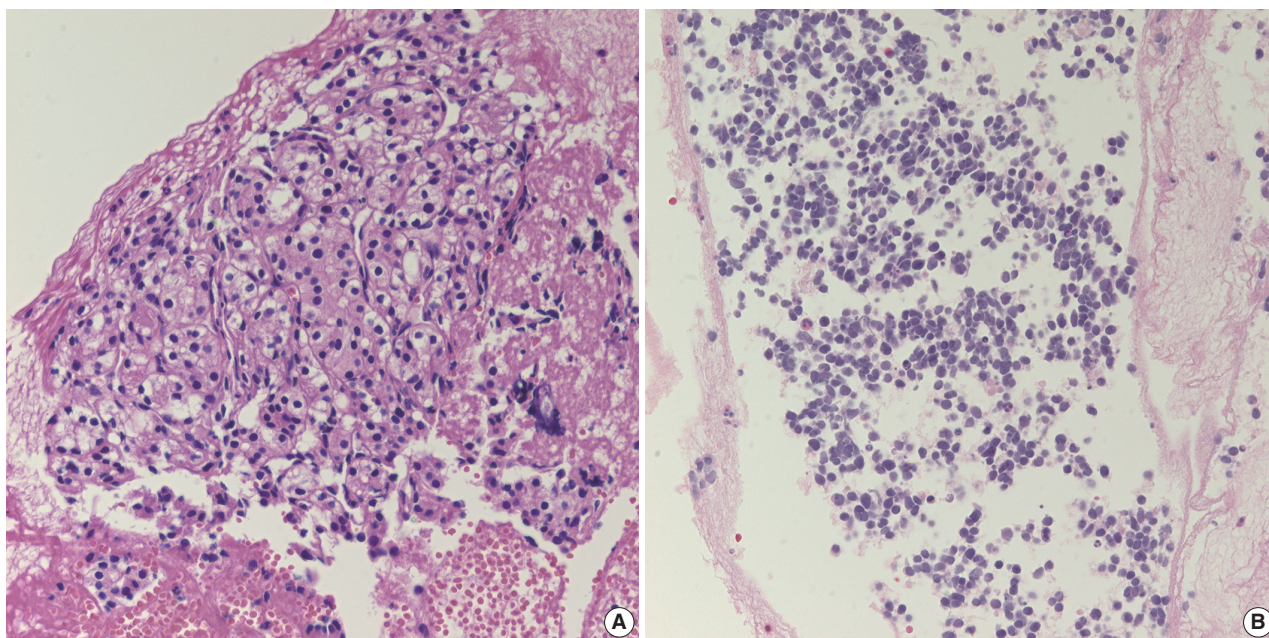


Fig. 6. (A) Metastatic renal cell carcinoma shows clear to granular cytoplasm on endoscopic ultrasound–guided fine needle aspiration (EUS-FNA) biopsy. (B) Metastatic small cell carcinoma from the lung exhibited discohesive small round cells with molding pattern on EUS-FNA biopsy.

ces may be evaluated to grade NETs in EUS-FNA aspirates. Although less frequently performed to diagnose ductal adenocarcinomas, immunohistochemical stains for p53 (overexpression or complete loss of expression), SMAD4 (loss of expression), and carcinoembryonic antigen (cytoplasmic expression) are useful in the differential diagnosis between ductal adenocarcinomas and benign reactive epithelial cells [11,20]. Fluorescence in situ hybridization for loss or alterations in copy number of 9p21, 3, 7, and 17 improves sensitivity for diagnosing ductal adenocarcinoma [21]. DNA sequencing, including next-generation sequencing, may also have a role in EUS-FNA diagnosis in the future. For example, testing for *KRAS* and *GNAS* mutations would help to discriminate between IPMN (*KRAS* or *GNAS* mutations in >96%), MCN (*KRAS* mutations but no *GNAS* mutations), and non-neoplastic cysts (e.g., pseudocyst), especially when there is only a scant amount of cells in the aspirated cyst fluid [11,22,23]. Molecular tests can be applied for differential diagnosis in pancreatic cystic fluid [24]. Some molecular panels are reportedly predictive in differential diagnosis of pancreatic cystic neoplasms. Furthermore, next-generation sequencing can be used in preoperative pathologic differential diagnosis of pancreas cystic lesions [25]. Molecular tests may be more widely used in pathologic evaluation of pancreas cystic lesions, which provide a very limited cellular component. Samples from EUS-FNA showed superior DNA quality than formalin-fixed paraffin-embedded tissue in some molecular analysis

[26]. However, any molecular test alone does not guarantee specific pathologic diagnosis, and they are more useful when used as supportive evidence rather than direct evidence of a specific disease entity. Practically, it is not uncommon to experience histopathological and immunohistochemical discrepancies between cytology/biopsy and resection specimens [27,28]. False negative results are more common on initial cytology/biopsy samples due to the heterogeneous histopathology and genomic profiles [29,30].

Clinical applications of molecular testing in practical pathologic diagnosis are limited because medical insurance reimbursement for molecular tests from EUS-FNA of various pancreatic lesions is not yet approved in Korea. Practical ancillary tests for EUS-FNA specimens in most Korean institutions are limited to immunohistochemical stains. However, when unresectable pancreatic cancer is being considered, more ancillary tests may be used because the EUS-FNA specimen is the only sample available to evaluate histopathologic and molecular characteristics of the tumor and provide information for precision medicine therapy.

HOW SHOULD PATHOLOGISTS CORRELATE CLINICAL AND/OR RADIOLOGIC FINDINGS WHEN INTERPRETING ENDOSCOPIC ULTRASOUND–GUIDED FINE NEEDLE ASPIRATION SPECIMENS?

EUS-FNA cytology/biopsy slides commonly demonstrate only

a few atypical cells that may be insufficient for a conclusive diagnosis of a malignancy. Therefore, it is often difficult for general surgical pathologists to confidently make a conclusive diagnosis on EUS-FNA specimens. In these cases, it may be better to adjust the threshold of malignancy according to the clinical and radiologic findings. For example, if a patient has multiple liver metastases and peritoneal seeding, the pathologist can suggest “malignancy” by lowering the threshold of malignancy, even with a few atypical cells on EUS-FNA. However, if the same cytology and/or biopsy is encountered in the setting of a clinical impression of “rule out autoimmune pancreatitis vs. hidden malignancy,” it may be better to provide a diagnosis of “atypical cells, uncertain malignant potential or cannot exclude malignancy.” Pathologists should raise their threshold of malignancy in such situations. Although this may be criticized as unprofessional, pathologists should be flexible to enable better communication with clinicians and improve patient management in diagnosis of EUS-FNA for pancreatic lesions. Clinical-pathologic correlation is essential in pathologic diagnosis of EUS-FNA from pancreatic lesions. If an EUS-FNA does not provide any atypical cells in a clinical setting of malignancy, pathologists should suggest the possibility of a non-representative sample in the pathology report. Suggested terminology is: “The possibility of non-representative sample remains. Re-aspiration is recommended to rule out malignancy.” Many clinicians think that a pathologic diagnosis of malignancy is difficult on EUS-FNA specimens. However, pathologic confirmation of a true negative is more difficult than confirmation of a true positive. Pathologists should be aware of inter-observer disagreement in radiologic interpretation of various pancreatic lesions. Due to the deep location of the pancreas, it is not feasible to obtain as many tumor cells as in cytology smears of the thyroid, breast, and uterine cervix. An open biopsy in the operation room would be required to obtain such quantities of tumor cells, which would be a burden to patients. In the era of molecular testing for precision medicine therapy, tumor volume is certainly an important issue. Given the small amounts of tumor cells obtained by EUS-FNA, more reliable sensitive molecular assays should be developed, such as circulating tumor DNA or mutant KRAS in circulating exosome [31,32]. Although there have been some advances in precision medicine strategies for pancreatic cancer, there are currently few targets for precision medicine in pancreatic tumors [33,34]. Until now, the main goal of the EUS-FNA cytology/biopsy is confirmation of a malignancy or non-neoplastic disease to guide decisions for the next step in therapy and patient management.

CONCLUSION

EUS-FNA cytology/biopsy for pancreatic lesions has become more routine in daily practice. Pathologists should be familiar with clinical and/or radiologic findings to make an accurate pathologic diagnosis of pancreatic lesions. Scant cellular smears or marked degeneration artifact are more likely from EUS-FNA for pancreatic lesions than for specimens of another organs. Pathologists should adjust their threshold of malignancy according to the clinical situation to avoid over- or under-treatment. Ancillary tests, including molecular analysis, can be helpful in differential diagnosis of EUS-FNA for various pancreatic lesions. However, they should be used as supportive evidence rather than direct diagnostic evidence in pathologic diagnosis of EUS-FNA.

Ethics Statement

Not applicable.

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Conflicts of Interest

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Prediction of *TP53* mutations by p53 immunohistochemistry and their prognostic significance in gastric cancer

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Background: Recently, molecular classifications of gastric cancer (GC) have been proposed that include *TP53* mutations and their functional activity. We aimed to demonstrate the correlation between p53 immunohistochemistry (IHC) and *TP53* mutations as well as their clinicopathological significance in GC. **Methods:** Deep targeted sequencing was performed using surgical or biopsy specimens from 120 patients with GC. IHC for p53 was performed and interpreted as strong, weak, or negative expression. In 18 cases (15.0%) with discrepant *TP53* mutation and p53 IHC results, p53 IHC was repeated. **Results:** Strong expression of p53 was associated with *TP53* missense mutations, negative expression with other types of mutations, and weak expression with wild-type *TP53* ($p < .001$). The sensitivity for each category was 90.9%, 79.0%, and 80.9%, and the specificity was 95.4%, 88.1%, and 92.3%, respectively. The TNM stage at initial diagnosis exhibited a significant correlation with both *TP53* mutation type ($p = .004$) and p53 expression status ($p = .029$). The Kaplan-Meier survival analysis for 109 stage II and III GC cases showed that patients with *TP53* missense mutations had worse overall survival than those in the wild-type and other mutation groups ($p = .028$). Strong expression of p53 was also associated with worse overall survival in comparison to negative and weak expression ($p = .035$). **Conclusions:** Results of IHC of the p53 protein may be used as a simple surrogate marker of *TP53* mutations. However, negative expression of p53 and other types of mutations of *TP53* should be carefully interpreted because of its lower sensitivity and different prognostic implications.

Key Words: Gastric cancer; p53; *TP53*; Next-generation sequencing; Immunohistochemistry

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TP53 is a tumor suppressor gene that encodes the protein p53, which is involved in cell cycle arrest in damaged cells that require DNA repair or in cases of damage beyond repair, triggering apoptosis. A defect in *TP53* is a crucial step in carcinogenesis. Previous studies noted that either a defect of the *TP53* gene itself or of a gene upstream or downstream of *TP53* was found in virtually all human cancers [1-3]. In gastric cancer (GC), p53 overexpression has been reported in 37.8%–54% of cases [4-6]. According to those studies, overexpression of p53 was generally associated with worse overall survival (OS) as well as well-known prognostic factors such as vascular invasion and lymph node metastasis.

In 2014, The Cancer Genome Atlas (TCGA) Research Net-

work Group proposed a molecular classification of GC [6]. The four subgroups were Epstein-Barr virus (EBV)-positive, microsatellite instability, genomic stability, and chromosomal instability. *TP53* alteration is a characteristic of the chromosomal instability group. In the following year, the Asian Cancer Research Group (ACRG) presented a different molecular classification that considered the three factors of microsatellite instability, epithelial-mesenchymal transition, and *TP53* mutation [7]. The four groups classified by those factors exhibited different prognoses. However, one of the limitations of those two studies was that the methodology used requires high-end and high-cost technologies such as next-generation gene sequencing. Different groups have attempted to develop a more practical imple-

mentation of the molecular classification of GC in clinical settings based on the biomarkers of TCGA and ACRG studies [8-10]. The immunohistochemistry (IHC) of p53 was used to practically predict the mutation status of *TP53*, but interpretation of p53 IHC was varied and has yet to be confirmed. Köbel et al. [11] demonstrated that optimal p53 IHC can accurately predict the mutation status of *TP53* in ovarian cancer, which can be very useful in diagnosis of high-grade serous carcinoma. This technique has yet to be validated for GC.

In this study, we aimed to measure the sensitivity, specificity, and accuracy of p53 IHC as a representation of *TP53* mutation status and to investigate the correlation between clinicopathologic features and p53 IHC or *TP53* mutations in GC. Therefore, we performed next-generation sequencing (NGS) and p53 IHC in 120 GC cases, and the *TP53* mutation statuses were compared with the p53 IHC results.

MATERIALS AND METHODS

Characterization of patients and sample acquisition

The study population was composed of 120 patients treated at Seoul National University Bundang Hospital (Seongnam, Korea) from 2009 to 2019. The median age was 60 years (range, 34 to 82 years), and 85 patients (70.8%) were men. Thirty-eight of the 120 cases (31.7%) were stage II at initial diagnosis, 71 (59.2%) cases were stage III, and 11 (9.2%) were stage IV. Among them, 109 stage II and III patients (90.8%) underwent curative radical resection (R0 resection) without preoperative chemotherapy or radiotherapy. In the 11 stage IV cases, endoscopic biopsy specimen was collected in one case, metastatectomy specimens in four cases, conversion surgery specimens after chemotherapy in five patients, and gastrectomy specimen in one case for the experiments. Analysis according to the World Health Organization (WHO) classification [12] revealed that tubular adenocarcinoma accounted for 54.2% (65 cases) of diagnoses, mucinous adenocarcinoma for 3.3% (4 cases), papillary adenocarcinoma for 3.3% (4 cases), poorly cohesive carcinoma for 30.0% (36 cases), and other minor histologic types for 9.2% (11 cases). For survival analysis, 109 patients with stage II and III GC were followed up from the date of surgery to the date of death or final follow-up. The median follow-up period was 42.2 months (range of 5.4-87.7 months).

Next-generation sequencing

Targeted sequencing of 170 cancer-related gene panels was performed using formalin-fixed, paraffin-embedded tissue (FFPE)

samples as previously described [13]. All FFPE materials had a short cold ischemic time not exceeding 2 hours, fixation time ranging from 8 to 72 hours, and were aged between 0 and 9 years.

In brief, approximately 3 µg of genomic DNA was extracted from FFPE tumor tissues, and the sequencing library was prepared using an Agilent SureSelect Target Enrichment Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's guidelines. High-throughput sequencing was performed using the HiSeq 2500 system (Illumina, San Diego, CA, USA) (Macrogen Inc., Seoul, Korea). After quality control of the FASTQ files, sequencing reads were aligned to the reference genome (GRCh37/hg19) using Burrows-Wheeler Aligner-MEM (BWA-MEM) [14]. Single nucleotide variants and small insertions and deletions (INDELs) were detected using the MuTect2 algorithm [15]. SnpEff and SnpSift v4.3i [16] with dbNSFP v2.9.3 [17] were used for variant annotation with various databases including the OncoKB [18] and ClinVar archives [19].

IHC staining

Immunohistochemical (IHC) staining for p53 (DO7, mouse monoclonal, Dako, Agilent Technologies) was performed on 3-µm-thick slides using an automated immunostainer (Benchmark XT, Ventana Medical Systems, Tucson, AZ, USA) following the manufacturer's protocol. The p53 IHC was interpreted in three tiers: strong nuclear staining in more than 10% of the tumor cells was considered strong positivity, samples without any nuclear staining of tumor cells (complete absence) were interpreted as negativity, and cases exhibiting weak, scattered, or patchy positivity were regarded as weak positivity. Representative images for each category are shown in Fig. 1. Cut-offs of 20% and 30% nuclear positivity were additionally applied for validation of the results.

For cases where gene mutation and protein expression status did not match (18 cases or 15.0%), p53 IHC was repeatedly performed and interpreted. In most cases (17 out of 18 or 94.4%), repeated immunohistochemical assays did not alter the initial interpretation. Tumor heterogeneity accounted for the change in one case. Initially, strong nuclear expression of p53 was observed in some areas of the tumor (< 10%) but was not sufficient to be classified as strong expression. Subsequent IHC was performed on another section of the same tumor, exhibiting overall strong expression of p53.

EBV in-situ hybridization

The EBV status was tested using EBV in-situ hybridization as previously described [20]. A fluorescein-conjugated EBV en-

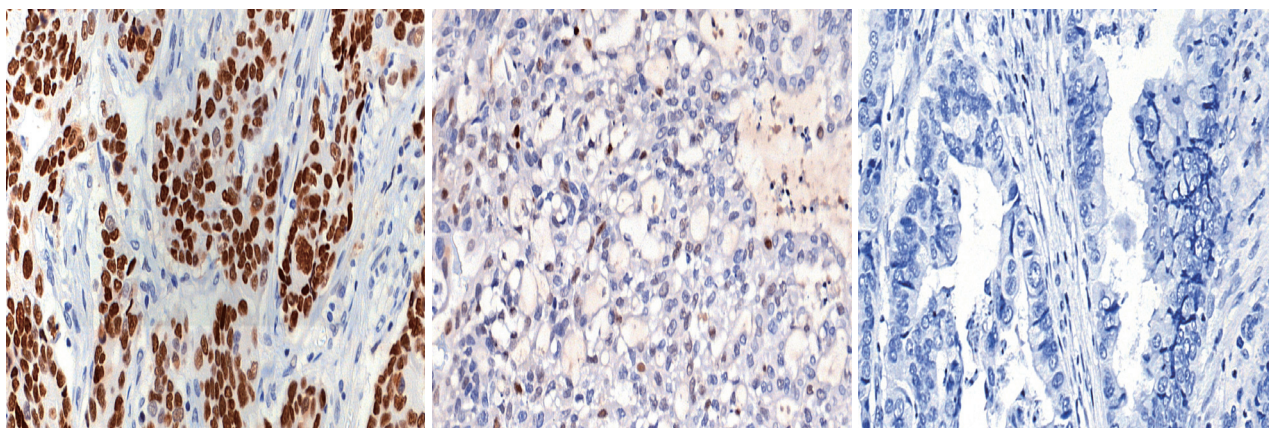


Fig. 1. Representative images of strong expression (A), weak expression (B), and loss of expression (C).

coded small RNA (EBER) oligonucleotide probe (INFORM EBVencoded RNA probe, Ventana Medical Systems) was used, and positive cases were defined as diffuse nuclear reactivity for EBER in tumor cells.

Microsatellite instability analysis

Representative tumor tissues and matched normal gastric mucosal tissues were selected for microsatellite instability (MSI) testing. Five NCI markers (BAT-26, BAT-25, D5S346, D17S250, and S2S123) amplified through polymerase chain reaction were analyzed using an automated sequencer (ABI 3731 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). MSI-high was defined as two or more markers with unstable peaks, MSI-low was defined as one unstable marker, and microsatellite stable was defined as no unstable marker.

Statistical analyses

Chi-square or Fisher exact tests were used to assess significant differences in the distribution of *TP53* mutations and p53 expression. For univariate survival analysis, Kaplan-Meier survival curves were plotted in 109 patients with stage II and III GC cases. The survival differences were compared using the log-rank test. For multivariate survival analysis, the Cox regression model was used. All statistical analyses were performed using SPSS Statistics ver. 22.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Gene mutation and protein expression correlation

Table 1 summarizes the p53 IHC results according to *TP53* mutations. *TP53* mutations were present in 52 cases (43.3%), of which missense mutations were the most common (33 of 52

Table 1. Comparison between *TP53* genetic mutations and p53 immunohistochemistry

<i>TP53</i> mutation	p53 expression by IHC			Total	p-value
	Strong	Negative	Weak		
Mutation status					<.001
Wild-type	1 (2.9)	12 (44.4)	55 (93.2)	68 (56.7)	
Mutation present	33 (97.1)	15 (55.6)	4 (6.8)	52 (43.3)	
Variant summary					<.001
Wild-type	1 (2.9)	12 (44.4)	55 (93.2)	68 (56.7)	
Missense	30 (88.2)	0	3 (5.1)	33 (27.5)	
Other	3 (8.9)	15 (55.6)	1 (1.7)	19 (15.8)	
Stop-gained	2 (5.9)	3 (11.1)	1 (1.7)	6 (5.0)	
Splice region	0	5 (18.5)	0	5 (4.2)	
Frameshift	0	7 (25.9)	0	7 (5.8)	
In-frame deletion	1 (2.9)	0	0	1 (0.8)	
Clinical significance ^a					<.001
Wild-type	1 (2.9)	12 (44.4)	55 (93.2)	68 (56.7)	
Pathogenic or likely pathogenic	22 (64.7)	13 (48.1)	2 (3.4)	37 (30.1)	
Uncertain significance	5 (14.7)	2 (7.4)	1 (1.7)	8 (6.7)	
Conflicting interpretation	6 (17.6)	0	1 (1.7)	7 (5.8)	
Total	34	27	59	120	

Values are presented as number (%).

^aAccording to the ClinVar and OncoKB databases accessed on March 18, 2020.

cases, 63.5%). Strong expression was observed in 34 cases (28.3%) and negative expression was observed in 27 cases (22.5%). When *TP53* mutations were compared with p53 IHC, 30 of the 33 missense mutation cases (90.9%) exhibited strong p53 expression, but negative expression of p53 was the dominant pattern (15 cases, 78.9%) among the 19 cases of other types of mutations ($p < .001$). Based on clinical significance, 37 cases (30.1%) had pathogenic or likely pathogenic *TP53* mutations, of which 22 cases (59.5%) exhibited strong expression of p53, 13 cases (36.1%) negative expression, and two cases (5.4%) weak ex-

pression ($p < .001$). Nevertheless, most cases of uncertain significance (62.5%) and conflicting interpretations (85.7%) also showed strong expression of p53 by IHC.

Detailed information about *TP53* mutations and p53 expression status is shown in Table 2. Two mutations were observed in three cases, of which one representative mutation was included in this table. One among seven cases with *TP53* mutations of conflicting interpretations regarding pathogenicity had weak expression of p53 (case No. 27 in Table 2). There have been reports suggestive of the “likely benign” and “uncertain significance” nature of this mutation. The mutations c.659A > G, c.742C > T, c.817C > T, c.796G > A, c.1024C > T, and c.375G > A were found in two cases, and c.818G > A mutation was found in three cases. The IHC results matched in cases with the same mutation. In 44 cases with single nucleotide polymorphism, C:G to T:A conversion was observed in 32 (72.7%), C:G to A:T in four (9.1%), C:G to G:C in two (4.5%), T:A to C:G in four (9.1%), and T:A to G:C in two (4.5%).

Sensitivity, specificity, and accuracy of p53 IHC for predicting *TP53* mutations

In general, nonsynonymous mutations detected using NGS were related to strong p53 expression in IHC. Similarly, all other types of mutations tended to show negative expression, of p53 while cases with wild-type *TP53* exhibited weak protein expression. The sensitivity of strong expression of p53 by IHC for predicting nonsynonymous *TP53* mutations was 90.9%, sensitivity of negative expression for other types of mutations was 79.0%, and the sensitivity of weak expression for wild-type *TP53* was 80.9% (Table 3). The specificity for each category was 95.4%, 88.1%, and 92.3%, respectively. The accuracy for each category was 94.2%, 86.7%, and 85.8%, respectively. In addition, the sensitivity, specificity, and accuracy of p53 IHC at 20% and 30% cut-offs are shown in Supplementary Table S1. The sensitivity of strong expression of p53 for nonsynonymous *TP53* mutations was highest at the 10% cut-off.

Clinicopathological variables and protein expression correlations

The correlation between clinicopathological characteristics and *TP53* mutations or p53 expression status is summarized in Table 4. TNM stage at initial diagnosis was the only variable that showed significant correlation with both *TP53* mutation type and p53 expression status ($p = .004$ and $p = .029$, respectively). Of the 38 stage II gastric cancer cases, 27 (71.1%) did not exhibit any detectable mutations in the *TP53* gene, but five

nonsynonymous (13.2%) and six other types of mutations (15.8%) were found. Strong p53 expression was found in seven of the 38 stage II cases (18.4%). Among the stage III cases, which accounted for 71 cases, the proportions of nonsynonymous gene mutations and strong expression of p53 mutations increased to 39.4% (28 cases) and 38.0% (27 cases), respectively. On the other hand, the proportions of wild-type *TP53* cases and weak expression cases decreased from 71.0% to 45.0% and from 55.2% to 43.6%, respectively.

TP53 mutations were more frequently observed in intestinal-type GC (25 of 45 cases, 55.6%) compared to the non-intestinal type (27 of 75 cases, 36.0%), but with borderline statistical significance ($p = .065$). Other clinicopathological variables such as sex, age, tumor location, and WHO classification were not statistically significant.

Survival analysis

One hundred nine patients with stage II and III GC at initial diagnosis were selected for survival analysis. The patients underwent curative surgery followed by adjuvant chemotherapy. Patients with any *TP53* mutations tended to have worse OS compared to those without mutations, although the difference was not statistically significant ($p = .227$). When OS was analyzed based on *TP53* mutation type, patients with nonsynonymous mutations had the worst OS, and the wild-type and other types of mutations exhibited similar OS ($p = .074$) (Fig. 2A). This trend became statistically significant when the nonsynonymous mutation group was compared to the combined wild-type and other mutation groups ($p = .028$) (Fig. 2B). The expression pattern of p53 was not significantly associated with patient OS ($p = .107$) (Fig. 2C), but it was statistically significant when strong expression of p53 was compared to the combined negative and weak expression cases ($p = .035$) (Fig. 2D). Patients with abnormal—negative and strong expression—expression did not exhibit a statistically significant survival difference compared to patients with weak expression ($p = .208$). The Kaplan-Meier survival curves of p53 expression status at 20% and 30% cut-offs were additionally plotted in Supplementary Fig. S1. The difference in survival was largest at the 30% cut-off. Multivariate Cox regression analysis showed that strong expression of p53 was associated with patient OS independent of stage with borderline significance ($p = .070$, data not shown). The presence of nonsynonymous missense mutations of *TP53* was not an independent prognostic factor in multivariate analysis ($p = .130$).

Table 2. Detailed information of *TP53* mutation and p53 expression status in gastric cancer patients with any *TP53* mutation

Case No.	Effect	Nucleic acid alteration	Amino acid alteration	Clinical significance ^a
1	Missense_variant	c.422G>A	p.Cys141Tyr	Pathogenic or likely pathogenic
2	Missense_variant	c.422G>T	p.Cys141Phe	Pathogenic or likely pathogenic
3	Missense_variant	c.455C>T	p.Pro152Leu	Pathogenic or likely pathogenic
4	Missense_variant	c.524G>A	p.Arg175His	Pathogenic or likely pathogenic
5	Missense_variant	c.535C>G	p.His179Asp	Pathogenic or likely pathogenic
6	Missense_variant	c.542G>A	p.Arg181His	Pathogenic or likely pathogenic
7	Missense_variant	c.659A>G	p.Tyr220Cys	Pathogenic or likely pathogenic
8	Missense_variant	c.659A>G	p.Tyr220Cys	Pathogenic or likely pathogenic
9	Missense_variant	c.701A>G	p.Tyr234Cys	Pathogenic or likely pathogenic
10	Missense_variant	c.725G>A	p.Cys242Tyr	Pathogenic or likely pathogenic
11	Missense_variant	c.734G>A	p.Gly245Asp	Pathogenic or likely pathogenic
12	Missense_variant	c.742C>T	p.Arg248Trp	Pathogenic or likely pathogenic
13	Missense_variant	c.742C>T	p.Arg248Trp	Pathogenic or likely pathogenic
14	Missense_variant	c.743G>A	p.Arg248Gln	Pathogenic or likely pathogenic
15	Missense_variant	c.772G>A	p.Glu258Lys	Pathogenic or likely pathogenic
16	Missense_variant	c.817C>T	p.Arg273Cys	Pathogenic or likely pathogenic
17	Missense_variant	c.817C>T	p.Arg273Cys	Pathogenic or likely pathogenic
18	Missense_variant	c.818G>A	p.Arg273His	Pathogenic or likely pathogenic
19	Missense_variant	c.818G>A	p.Arg273His	Pathogenic or likely pathogenic
20	Missense_variant	c.818G>A	p.Arg273His	Pathogenic or likely pathogenic
21	Missense_variant	c.380C>T	p.Ser127Phe	Conflicting interpretations of pathogenicity
22	Missense_variant	c.473G>C	p.Arg158Pro	Conflicting interpretations of pathogenicity
23	Missense_variant	c.481G>A	p.Ala161Thr	Conflicting interpretations of pathogenicity
24	Missense_variant	c.613T>C	p.Tyr205His	Conflicting interpretations of pathogenicity
25	Missense_variant	c.796G>A	p.Gly266Arg	Conflicting interpretations of pathogenicity
26	Missense_variant	c.796G>A	p.Gly266Arg	Conflicting interpretations of pathogenicity
27	Missense_variant	c.1015G>A	p.Glu339Lys	Conflicting interpretations of pathogenicity
28	Missense_variant	c.329G>A	p.Arg110His	Uncertain significance
29	Missense_variant	c.380C>A	p.Ser127Tyr	Uncertain significance
30	Missense_variant	c.476C>T	p.Ala159Val	Uncertain significance
31	Missense_variant	c.797G>T	p.Gly266Val	Uncertain significance
32	Missense_variant	c.400T>G	p.Phe134Val	Uncertain significance
33	Missense_variant	c.470T>G	p.Val157Gly	Uncertain significance
34	Frameshift_variant	c.331_332insAG	p.Leu111fs	Pathogenic or likely pathogenic
35	Frameshift_variant	c.381_391delCCCTGCCCTCA	p.Pro128fs	Pathogenic or likely pathogenic
36	Frameshift_variant	c.635_669delTTTCGACATAGTGTGGTG GTGCCCTATGAGCCGCCT	p.Phe212fs	Pathogenic or likely pathogenic
37	Frameshift_variant	c.660_661delTG	p.Tyr220fs	Pathogenic or likely pathogenic
38	Frameshift_variant	c.747delG	p.Arg249fs	Pathogenic or likely pathogenic
39	Frameshift_variant	c.1169delC	p.Pro390fs	Pathogenic or likely pathogenic
40	Frameshift_variant	c.778_779delTTC	p.Ser260fs	Uncertain significance
41	Conservative_inframe_deletion	c.529_546delCCCCACCATGAGCGCTGC	p.Pro177_Cys182del	Pathogenic or likely pathogenic
42	Stop_gained	c.159G>A	p.Trp53*	Pathogenic or likely pathogenic
43	Stop_gained	c.437G>A	p.Trp146*	Pathogenic or likely pathogenic
44	Stop_gained	c.586C>T	p.Arg196*	Pathogenic or likely pathogenic
45	Stop_gained	c.637C>T	p.Arg213*	Pathogenic or likely pathogenic
46	Stop_gained	c.1024C>T	p.Arg342*	Pathogenic or likely pathogenic
47	Stop_gained	c.1024C>T	p.Arg342*	Pathogenic or likely pathogenic
48	Splice_region_variant&synonymous_variant	c.375G>A	p.Thr125Thr	Pathogenic or likely pathogenic
49	Splice_region_variant&synonymous_variant	c.375G>A	p.Thr125Thr	Pathogenic or likely pathogenic
50	Splice_region_variant&synonymous_variant	c.375G>C	p.Thr125Thr	Pathogenic or likely pathogenic
51	Splice_acceptor_variant&intron_variant	c.920 - 1G>A		Pathogenic or likely pathogenic
52	Splice_donor_variant&intron_variant	c.96 + 1G>A		Uncertain significance (no report)

IHC, immunohistochemistry.

^aAccording to the ClinVar and OncoKB databases accessed on March 18, 2020.

Table 3. The sensitivity, specificity, and accuracy of p53 immunohistochemistry for predicting *TP53* mutation, cut-off 10%

<i>TP53</i> mutation	Sensitivity (%)	Specificity (%)	Accuracy (%)
Nonsynonymous mutation by p53 strong expression	90.9	95.4	94.2
Other type mutation by negative expression of p53	79.0	88.1	86.7
Wild-type by weak expression of p53	80.9	92.3	85.8

Table 4. Clinicopathologic characteristics according to *TP53* mutation and p53 expression status

Characteristic	Total	<i>TP53</i> mutation				p53 expression			
		NS	Other	Wild	p-value	Strong	Negative	Weak	p-value
No.	120	33	19	68		34	27	59	
Age (yr)					0.248				0.470
<65	69 (57.5)	15 (45.5)	11 (57.9)	43 (63.2)		17 (50.0)	15 (55.6)	37 (62.7)	
≥65	51 (42.5)	18 (54.5)	8 (42.1)	25 (36.8)		17 (50.0)	12 (44.4)	22 (37.3)	
Sex					0.117				0.285
Male	85 (70.8)	27 (81.8)	15 (78.9)	43 (63.2)		27 (79.4)	20 (74.1)	38 (64.4)	
Female	35 (29.2)	6 (18.2)	4 (21.1)	25 (36.8)		7 (20.6)	7 (25.9)	21 (35.6)	
Location of tumor center					0.940				0.856
Lower third	53 (44.2)	15 (45.5)	10 (52.6)	28 (41.2)		16 (47.1)	11 (40.7)	26 (44.1)	
Middle third	33 (27.5)	9 (27.3)	4 (21.1)	20 (29.4)		7 (20.6)	8 (29.6)	18 (30.5)	
Upper third	34 (28.3)	9 (27.3)	5 (26.3)	20 (29.4)		11 (32.4)	8 (29.6)	15 (25.4)	
TNM at initial diagnosis					0.004				0.029
II	38 (31.7)	5 (15.2)	6 (31.6)	27 (39.7)		7 (20.6)	10 (37.0)	21 (35.6)	
III	71 (59.2)	28 (84.8)	11 (57.9)	32 (47.1)		27 (79.4)	13 (48.1)	31 (52.5)	
IV	11 (9.2)	0	2 (10.5)	9 (13.2)		0	4 (14.8)	7 (11.9)	
WHO classification					0.733				0.596
Papillary	4 (3.3)	1 (3.0)	1 (5.3)	2 (2.9)		1 (2.9)	2 (7.4)	1 (1.7)	
Tubular WD/MD	28 (23.3)	10 (30.3)	6 (31.6)	12 (17.6)		10 (29.4)	6 (22.2)	12 (20.3)	
Tubular PD	37 (30.8)	9 (27.3)	7 (36.8)	21 (30.9)		10 (29.4)	8 (29.6)	19 (32.2)	
PCC	36 (30.0)	8 (24.2)	3 (15.8)	25 (36.8)		7 (20.6)	7 (25.6)	22 (37.3)	
Mucinous	4 (3.3)	2 (6.1)	0	2 (2.9)		2 (5.9)	1 (3.7)	1 (1.7)	
Others	11 (9.2)	3 (9.1)	2 (10.5)	6 (8.8)		4 (11.7)	3 (11.1)	4 (6.8)	
Lauren classification					0.065				0.587
Intestinal	45 (37.5)	14 (42.4)	11 (57.9)	20 (29.4)		15 (44.1)	10 (37.0)	20 (33.9)	
Non-intestinal	75 (62.5)	19 (57.6)	8 (42.1)	48 (70.6)		19 (55.9)	17 (63.0)	39 (66.1)	
EBV					0.215				0.036
Negative	105 (87.5)	31 (93.9)	18 (94.7)	56 (82.4)		30 (88.2)	27 (100)	48 (81.4)	
Positive	15 (12.5)	2 (6.1)	1 (5.3)	12 (17.6)		4 (11.8)	0	11 (18.6)	
MSI					0.258				0.010
MSS/MSI-L	112 (93.3)	32 (97.0)	19 (100)	61 (89.7)		34 (100)	27 (100)	51 (86.4)	
MSI-H	8 (6.7)	1 (3.0)	0	7 (10.3)		0	0	8 (13.6)	

Values are presented as number (%).

NS, nonsynonymous; Other, other type mutation; wild, wild-type; WHO, World Health Organization; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; PCC, poorly cohesive carcinoma; EBV, Epstein-Barr virus; MSI, microsatellite instability; MSS, microsatellite stable; MSI-L, microsatellite instability-low; MSI-H, microsatellite instability-high.

DISCUSSION

TP53 is the most well-known tumor suppressor gene, and p53 IHC is a method used in daily practice as a surrogate marker in various cancer patients. In this study, we performed targeted deep sequencing for detecting various *TP53* mutations and IHC for p53 using a commercially available and validated primary antibody with an automatic immunostainer. Strong expression

of p53 could predict nonsynonymous missense mutations of *TP53* with a sensitivity of 90.9%, specificity of 95.4%, and accuracy of 94.2%. However, weak expression of p53 was less specific (80.9%) for predicting wild-type *TP53*, and negative expression was less sensitive (79.0%) for predicting other mutations of *TP53*. These results suggest that p53 IHC can be used as a surrogate marker in predicting *TP53* mutations, especially for strong expression, to predict nonsynonymous mutations. There

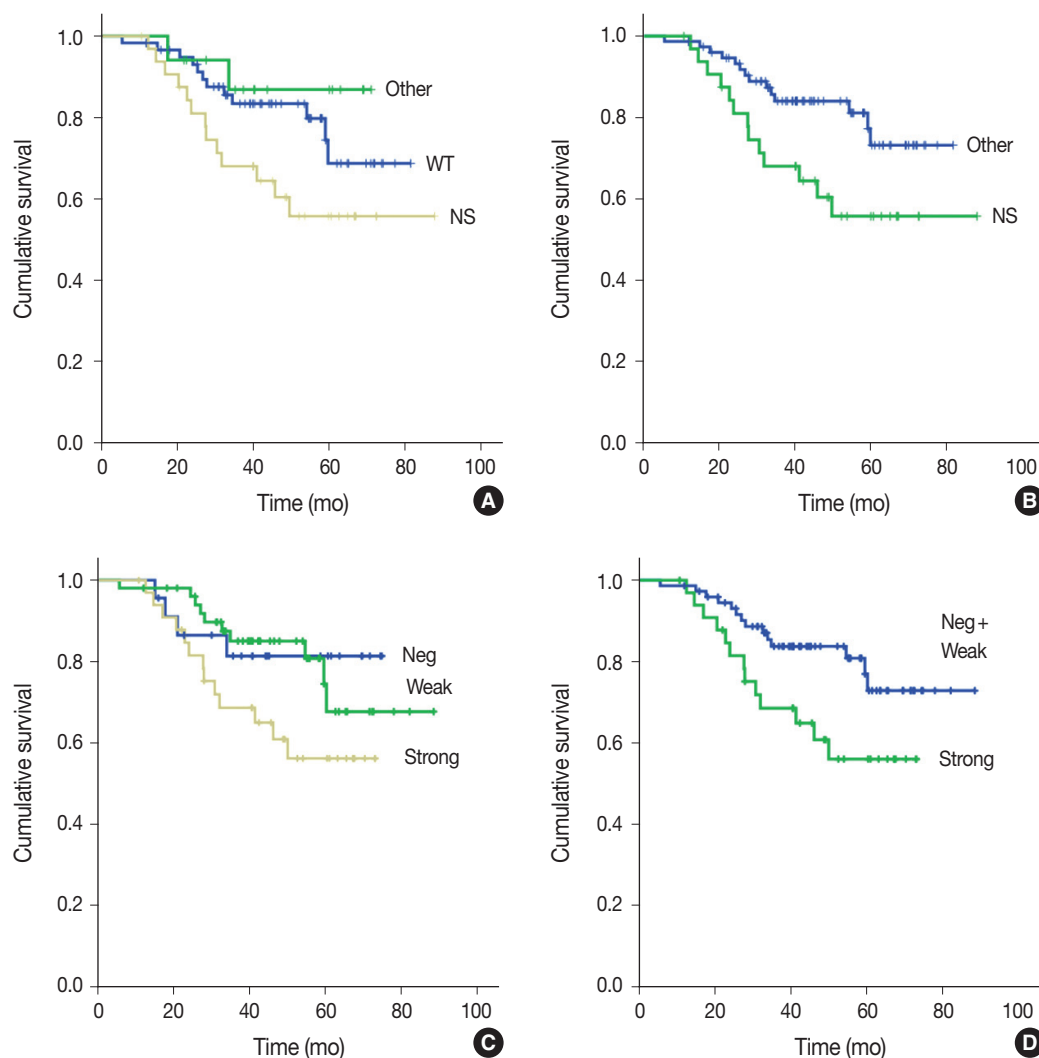


Fig. 2 . Kaplan-Meier survival curves of cumulative survival rate versus follow-up months after surgery according to mutation status (A, B) and immunohistochemistry results (C, D). (A) Nonsynonymous mutations (NS, beige) versus wild-type (WT, green) versus other types of mutations (other, green) ($p = .074$). (B) Nonsynonymous mutations (NS, green) versus combined wild-type and other types of mutations (other, blue) ($p = .028$). (C) Strong (beige) versus weak (green) versus negative expression (Neg) ($p = .107$). (D) Strong (green) versus combined weak and negative expression (Neg + Weak, blue) ($p = .035$).

have been recent attempts to use IHC for molecular classifications, and their clinicopathological significance has been increasingly important in GC [8,10]. Our results will be helpful for these new molecular classifications although negative expression should be cautiously interpreted.

Based on the Kaplan-Meier survival analysis, strong expression of p53 was significantly associated with worse OS compared to weak and negative expression of p53 in this study. Previous studies that investigated any relationship between p53 overexpression and survival reported p53 overexpression as a poor prognostic factor [21-23], similar to the findings from our study. Some studies did not reveal the prognostic significance of p53 overex-

pression in GC, but a meta-analysis demonstrated that it is a poor prognostic factor [24]. In those studies, the median cut-off value was 10% [24]. Therefore, we applied a cut-off value of 10% for defining strong expression of p53. In addition to the 10% cut-off, we applied 20% and 30% cut-offs in this study. Although the survival difference was largest at the 30% cut-off, the sensitivity of strong expression of p53 for predicting nonsynonymous *TP53* mutation was highest at the 10% cut-off. Therefore, further studies are needed to validate various cut-offs.

For interpretation of p53 IHC, Köbel et al. [11,25] proposed a three-tiered scoring system, including overexpression, complete absence, and normal or wild-type pattern in ovarian cancer. The

scoring system exhibited good correlation with *TP53* mutation status: overexpression with nonsynonymous mutation; complete absence with stop gain, frameshift, and splicing mutations; and a normal pattern with the wild-type *TP53* gene [11]. Shin et al. [26] investigated the prognostic roles of p53 expression status in patients with GC. They defined group 0 as complete absence, group 1 as weak staining in < 50%, group 2 as strong staining in 50%–90%, and group 3 as strong staining in > 90%. When the Kaplan-Meier survival analysis was performed, group 1 was associated with better survival than groups 0, 2, and 3, but with borderline statistical significance. Our results showed a similar relationship between p53 IHC and *TP53* mutation status to those of previous studies. If weak expression in this study was defined as a normal or wild-type pattern, the Kaplan-Meier survival curves did not show a significant difference between normal and abnormal expression patterns. Furthermore, patients with GC having nonsynonymous *TP53* mutations had significantly worse prognosis compared to patients with other types of mutations and the wild-type *TP53* group. Similarly, strong expression of p53, which was related to nonsynonymous *TP53* mutations, was shown to be a poor prognostic factor. The complete absence of p53 expression or other types of *TP53* mutations might not be significant for predicting prognosis.

There were 17 cases (14.2%) with discrepant results between p53 IHC and *TP53* mutations. Most discrepant cases had negative expression of p53 and wild-type *TP53*. Weak expression of p53 was observed in four missense mutation cases and one stop gain mutation case. These findings might be due to tumor heterogeneity or tissue quality issues, such as specimen ischemic time or archival age. In addition, there was one case with weak p53 expression and a missense mutation of conflicting pathogenic interpretation. Considering this case was conflicting between uncertain significance and likely benign significance, one of the possible reasons for the discrepancy is a non-pathogenic mutation. To discriminate functional and nonfunctional p53, Nenutil et al. [27] performed IHC for p53, Ki67, MDM2, and p21 in human cancers, and overexpressed p53 without increased MDM2 indicated inactivating mutations in their study. p21, a transcriptional target of p53, was considered to reflect p53 activity and could decrease false-positive results of p53 expression [28]. The ACRG considered a *TP53*-activity signature using p21 and MDM2 genes [7]. Therefore, in addition to p53 IHC, IHC for p21 and MDM2 would be helpful for evaluating the functional status of p53. The need for these additional analyses reflects a potential limitation of this study, necessitating further research.

In a total of 120 gastric cancer cases, 52 (43.3%) had *TP53* mutations, including nonsynonymous missense, frameshift, stop gain, in-frame deletion, and splice region mutations. Hot spot mutations within the central core (R175, G245, R248, R273, and R282) were observed in a minority of cases (10 out of 120 or 8.3%). In accordance with our results, a previous study reported hot spot mutations in 6.2% of gastric cancer cases [29]. Therefore, sequencing (Sanger or NGS) is suggested as a suitable method for detecting *TP53* mutations in gastric cancer.

In summary, we investigated the relationship between p53 expression and *TP53* mutation status to predict *TP53* mutations by p53 IHC and reveal their prognostic significance. *TP53* mutations were observed in 43.3% of cases. Strong p53 expression could predict nonsynonymous missense mutations with high sensitivity and specificity, but only half of the p53 negative cases (55.6%) exhibited other types of *TP53* mutations. Protein overexpression and nonsynonymous genetic mutations of *TP53* significantly predicted worse OS. p53 IHC could be regarded as a simple surrogate marker of *TP53* mutations, but negative expression of p53 and other types of *TP53* mutations should be cautiously considered in daily practice or scientific research. Overall, our study results will be informative for simple molecular classification of patients with GC.

Supplementary Information

The Data Supplement is available with this article at <https://doi.org/10.4132/jptm.2020.06.01>.

Ethics Statement

This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (IRB number: B-2001/591-105). Written informed consent was waived by the IRB.

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Conflicts of Interest

W.H.K. and H.S.L., contributing editors of the *Journal of Pathology and Translational Medicine*, were not involved in the editorial evaluation or decision to publish this article. All remaining authors have declared no conflicts of interest.

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Lymph node size and its association with nodal metastasis in ductal adenocarcinoma of the pancreas

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Background: Although lymph node metastasis is a poor prognostic factor in patients with pancreatic ductal adenocarcinoma (PDAC), our understanding of lymph node size in association with PDAC is limited. Increased nodal size in preoperative imaging has been used to detect node metastasis. We evaluated whether lymph node size can be used as a surrogate preoperative marker of lymph node metastasis. **Methods:** We assessed nodal size and compared it to the nodal metastatic status of 200 patients with surgically resected PDAC. The size of all lymph nodes and metastatic nodal foci were measured along the long and short axis, and the relationships between nodal size and metastatic status were compared at six cutoff points. **Results:** A total of 4,525 lymph nodes were examined, 9.1% of which were metastatic. The mean size of the metastatic nodes (long axis, 6.9 ± 5.0 mm; short axis, 4.3 ± 3.1 mm) was significantly larger than that of the non-metastatic nodes (long axis, 5.0 ± 4.0 mm; short axis, 3.0 ± 2.0 mm; all $p < .001$). Using a 10 mm cutoff, the sensitivity, specificity, positive predictive value, overall accuracy, and area under curve was 24.8%, 88.0%, 17.1%, 82.3%, and 0.60 for the long axis and 7.0%, 99.0%, 40.3%, 90.6%, and 0.61 for the short axis, respectively. **Conclusions:** The metastatic nodes are larger than the non-metastatic nodes in PDAC patients. However, the difference in nodal size was too small to be identified with preoperative imaging. The performance of preoperative radiologic imaging to predict lymph nodal metastasis was not good. Therefore, nodal size cannot be used a surrogate preoperative marker of lymph node metastasis.

Key Words: Pancreas; Neoplasms; Lymph node; Size; Metastasis

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Pancreatic cancer, the most common subtype of which is pancreatic ductal adenocarcinoma (PDAC), is the leading cause of cancer-related death in the United States and Korea [1-3]. In 2017, an estimated 448,000 people were diagnosed with PDAC and 441,000 people died from the disease worldwide. Only 10% of patients with PDAC are in an early stage of disease at the time of diagnosis [3]. Most patients with PDAC are diagnosed with advanced stage disease with regional extension and distant metastasis at the time of diagnosis. Therefore, most patients are not eligible for surgical resection [4].

Lymph node metastasis is a poor prognostic factor in patients with PDAC [5-8]. Nodal status and quantity are important, and nodal (N) staging is classified based on the number of metastatic

lymph nodes, as N0 (0), N1 (1–3), and N2 (≥ 4), in the 8th edition of the American Joint Committee on Cancer [9]. Several other aspects of lymph node metastasis, including the lymph node ratio, are strong prognostic indicators [10]. A minimum of 12 lymph nodes should be examined to properly evaluate the N0 category [11,12].

Imaging is important for the preoperative staging of PDACs. Lymph nodes with a short axis ≥ 1 cm or an abnormal shape, including a round contour, irregular margin, and hypodense or heterogeneous density, are suspicious for metastasis [9]. However, it is difficult to assess metastatic lymph nodes by imaging such as computed tomography (CT) or magnetic resonance imaging (MRI), as the nodes may be enlarged due to metastasis or reactive

hyperplasia from known or unknown stimuli [13]. Enlarged peripancreatic lymph nodes may indicate nodal metastasis during surgical resection of the PDAC. The surgeon may sample the enlarged lymph node for intraoperative pathologic consultation with frozen sectioning.

Previous research compared lymph node size based on metastatic status and concluded that lymph node size was not a good indicator for metastatic involvement [14-21]. However, the number of cases included in these studies was too small to make a solid conclusion. Therefore, a validation study is required. To evaluate whether lymph node size can be used as a surrogate preoperative marker of lymph node metastasis, we systematically assessed lymph node size based on nodal metastasis in 200 surgically resected PDACs.

MATERIALS AND METHODS

Case selection

A total of 223 consecutive surgically resected PDAC cases from December of 2013 to December of 2014 were selected from a pathology archive. PDAC was the only pancreatic cancer included because it made up most of the surgically resected pancreatic cancer cases. Other histologic subtypes, such as adenosquamous, colloid, medullary, undifferentiated carcinomas, undifferentiated carcinomas with osteoclast-like giant cells, and squamous cell carcinomas, were not present. Fifteen PDACs arising from intraductal papillary mucinous neoplasm and one double primary cancer with concurrent pancreatic duodenal cancer were excluded. In addition, seven patients who had preoperative chemo-radiation therapy were also excluded. Finally, a total of 200 PDAC cases, including 133 pancreaticoduodenectomy, 58 distal pancreatectomy, and nine total pancreatectomy cases, were selected, and all cases had lymph node dissection. A flow chart illustrating the inclusion and exclusion criteria is depicted in Fig. 1. Clinical characteristics such as age and sex of the patient, tumor location, operation type, follow-up data, survival data, and recurrence and metastasis data were obtained from the electronic medical records. Pathologic data were evaluated, i.e., tumor size, invasion into the extrahepatic bile duct, duodenum, and spleen, histologic tumor grade, lymphovascular and perineural invasion, invasion of the large vessel, including splenic, portal, or superior mesenteric veins, the cancer resection margin, and pT and pN categories. The pT and pN categories were determined based on the 8th edition of the American Joint Committee on Cancer cancer staging system [9].

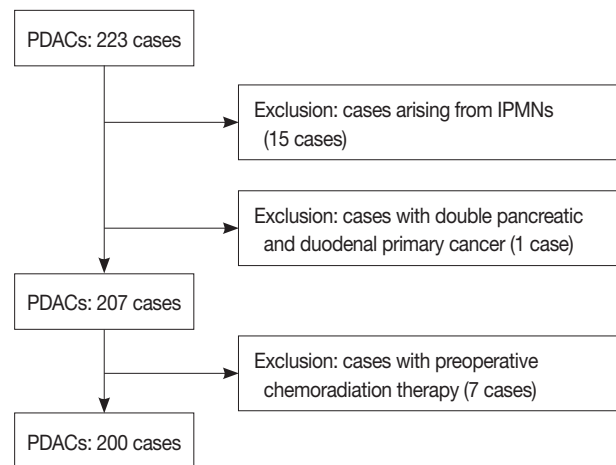


Fig. 1. Flow chart for patient selection. PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary mucinous neoplasm.

Radiologic evaluation of suspicious metastatic lymph nodes

Lymph nodes suspicious for metastasis were defined as those having a short axis more than 10 mm or an abnormal shape, including a round contour with an irregular margin or hypodense or heterogeneous density [22].

Histopathological evaluation of lymph nodes

Lymph nodes were meticulously retrieved during gross examination. Small lymph nodes were submitted as a single piece, while large lymph nodes were bisected [23]. Six cases were randomly selected to evaluate the shrinkage effect during the overnight fixation procedure with 10% neutral buffered formalin. The size of 85 lymph nodes was measured at the time of gross examination (before fixation) and on the formalin-fixed paraffin-embedded (FFPE) tissue blocks (after fixation). The size of the lymph nodes both in the long and short axes on hematoxylin and eosin (H&E) slides was measured. Representative gross (before and after fixation) and microscopic images of lymph node are depicted in Fig. 2.

All H&E stained slides were carefully reviewed by two pathologists (J.S. and S.M.H.). The total examined and metastatic lymph nodes were counted. Since most pathologic examinations measure and use the largest dimension, the dimension of the long axis of each lymph node (in mm) was measured with a ruler and recorded, regardless of metastatic status. In addition, the dimension of the short axis of each lymph node was also measured, because measurement of the short axis of lymph nodes has been used in preoperative radiologic evaluation [22]. The largest dimension of each metastatic foci was measured and recorded. The lymph node size distribution and the relationship between lymph node size and metastatic status were evaluated.

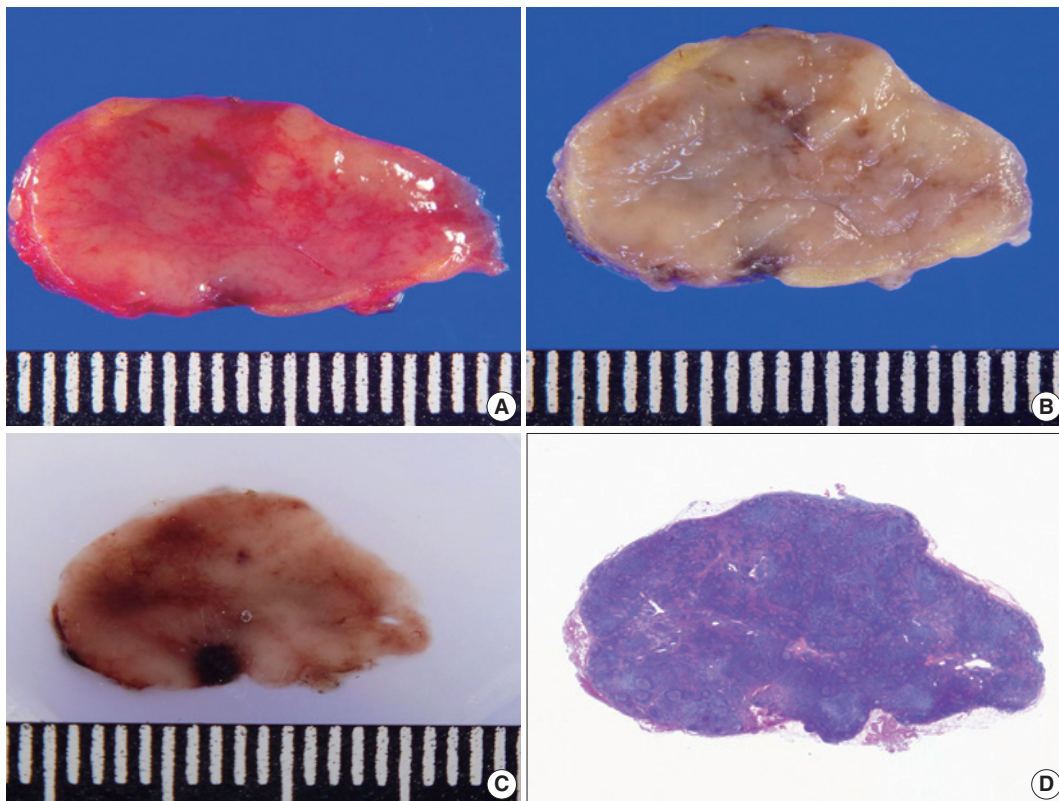


Fig. 2. Representative gross (before and after fixation) and microscopic images of evaluated lymph nodes. Gross images of lymph node before (A) and after (B) fixation. (C) Image of lymph node in formalin fixed paraffin embedded tissue block. (D) Microscopic image on H&E stained slides of lymph node.

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, ver. 20.0 (IBM Corp., Armonk, NY, USA) and R project ver. 3.6.1. Categorical variables were compared by the Pearson chi-square and Fisher exact tests. Continuous variables were compared by the independent Student's t-test and Wilcoxon signed-rank test. A p-value less than .05 was considered statistically significant. We used a logistic regression model to predict the probability of lymph node metastasis. Receiver operating characteristic (ROC) curves were generated to calculate the area under the curve (AUC), which measures the predictive power of lymph node size for estimating metastasis by preoperative imaging modalities.

RESULTS

Case characteristics

The clinicopathologic characteristics of the patients are summarized in Table 1. The mean patient age was 62.1 ± 10.0 years (range, 35 to 82 years), and 113 were male and 87 were female. The mean tumor size was 33 ± 13 mm (range, 6 to 88 mm). Ap-

proximately two-thirds (113) of the cases were classified as T2 (66.5%), 22 cases (11%) were T1, 37 cases (18.5%) were T3, and eight cases (4%) were classified as T4. Lymphovascular and perineural invasion occurred in 149 (74.5%) and 181 (90.5%) cases, respectively. Lymph node metastasis was observed in 124 (62%) cases. Of the cases with nodal metastasis, 85 were N1 cases (42.5%) and 39 were N2 cases (19.5%). The median follow-up period was 20 months (range, 1 to 71 months), and 161 of 200 patients died during this period.

Comparison of lymph node size before and after fixation

The mean long axis nodal sizes of 85 lymph nodes from six randomly selected cases before and after the fixation procedure were 6.9 ± 4.5 mm (range, 2 to 19 mm) and 6.5 ± 3.9 mm (range, 2 to 17 mm), respectively. There was a significant decrease in lymph node size after fixation (Wilcoxon signed-rank test, $p < .001$). The calculated shrinkage effect (mean, 0.47 ± 0.75 ; 95% confidence interval [CI], 0.31 to 0.63) after fixation was 6.8%. There were 14 metastatic (16%) and 71 were non-metastatic (84%) lymph nodes. The mean sizes of metastatic nodes along the long axis before and after fixation were 8.7 ± 5.2 mm and 8.1 ± 4.8 mm,

Table 1. The clinicopathologic characteristics of 200 patients with surgically resected PDAC based on nodal metastasis status

Characteristic	Without lymph node metastasis	With lymph node metastasis	p-value
No. of patients	76	124	
Age (yr)	62.7 ± 1.1 (43–81)	61.8 ± 0.9 (35–82)	.544
Sex			.986
Male	43 (56.6)	70 (56.5)	
Female	33 (43.4)	54 (43.5)	
Peripancreatic soft tissue invasion	71 (93.4)	122 (98.4)	.107
Common bile duct invasion	32 (42.1)	67 (54.0)	.102
Duodenum invasion	27 (35.5)	57 (46.0)	.146
Large vessel invasion			.007
Absent	65 (85.5)	85 (68.5)	
Present	11 (14.5)	39 (31.5)	
Surgical margin status			.027
Free	57 (75.0)	74 (59.7)	
Involved	19 (25.0)	50 (40.3)	
Tumor size (cm)	3.0 ± 0.1 (0.6–7.0)	3.4 ± 0.1 (1.3–8.8)	.015
Tumor location			.338
Head/uncinate/neck	49 (64.5)	81 (65.3)	
Body/tail	23 (30.3)	30 (24.2)	
Diffuse or multifocal	4 (5.3)	13 (10.5)	
T category			.008
pT1	13 (17.1)	9 (7.3)	
pT2	53 (69.7)	80 (64.5)	
pT3	10 (13.2)	27 (21.8)	
pT4	0	8 (6.5)	
Histologic grade			.188
Well differentiated	5 (6.6)	2 (1.6)	
Moderately differentiated	60 (78.9)	102 (82.3)	
Poorly differentiated	11 (14.5)	20 (16.1)	
Lymphovascular invasion			< .001
Absent	34 (44.7)	17 (13.7)	
Present	42 (55.3)	107 (86.3)	
Perineural invasion			.167
Absent	10 (13.2)	9 (7.3)	
Present	66 (86.8)	115 (92.7)	

Values are presented as number (%) or mean ± SD (range).

PDAC, pancreatic ductal adenocarcinoma; SD, standard deviation.

respectively. The mean sizes of non-metastatic nodes along the long axis before and after fixation were 6.6 ± 4.3 mm and 6.1 ± 3.7 mm, respectively. No significant differences in the shrinkage effect were observed between the metastatic and non-metastatic lymph node groups ($p = .585$).

The mean short axis nodal sizes before and after the fixation procedure were 4.2 ± 2.6 mm (range, 1 to 15 mm) and 3.9 ± 2.2 mm (range, 1 to 13.5 mm), respectively. There was a significant decrease in lymph node size after fixation (Wilcoxon signed-rank test, $p < .001$). The calculated shrinkage effect (mean, 0.33 ± 0.49 ; 95% CI, 0.22 to 0.44) after fixation was 7.9%. The mean sizes of metastatic nodes along the short axis before and after fixation were 5.4 ± 3.6 mm and 4.9 ± 3.1 mm, respectively. The

mean sizes of non-metastatic nodes along the short axis before and after fixation were 4.0 ± 2.4 mm and 3.7 ± 2.0 mm, respectively. No significant differences in the shrinkage effect were observed between the metastatic and non-metastatic lymph node groups ($p = .156$).

Association of lymph node metastasis and other clinicopathologic factors

The associations between lymph node metastasis and other clinicopathologic factors are summarized in Table 1. Lymph node metastasis was associated with large tumor size ($p = .015$), invasion into large vessels, including the splenic, portal, and/or superior mesenteric veins ($p = .007$), involvement of the resection

margin ($p = .027$), high T categories ($p = .008$), and lymphovascular invasion ($p < .001$).

Comparison of lymph node size based on metastatic status

A total of 4,525 lymph nodes were evaluated in this study; 412 (9.1%) had metastatic foci, while 4,113 (90.9%) had no metastasis. The mean number of examined lymph nodes was 22.6 ± 11.9 per case (Fig. 3). The mean number of metastatic lymph nodes was 2.1 ± 2.6 per case (Fig. 4). The mean sizes of the long and short axes of all lymph nodes were 5.2 ± 4.1 mm and 3.1 ± 2.2 mm, respectively. The mean size of the long axis of the 412

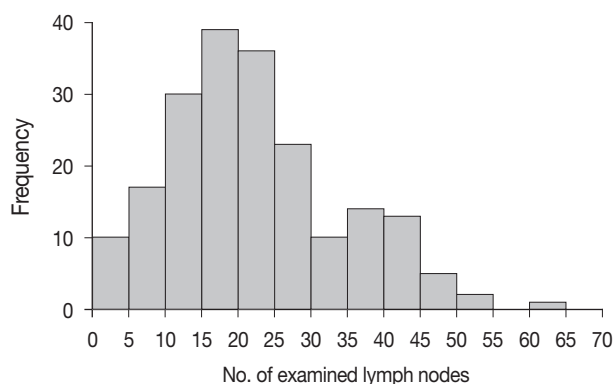


Fig. 3. Examined lymph node distribution, per case.

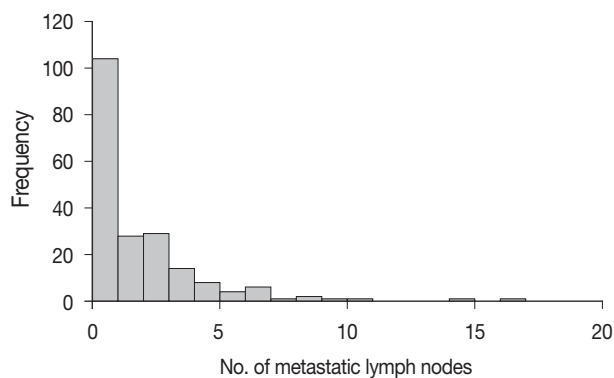


Fig. 4. Metastatic lymph node distribution, per case.

metastatic lymph nodes (6.9 ± 5.0 mm) was significantly larger than that of the non-metastatic lymph nodes (5.0 ± 4.0 mm, $p < .001$). Similarly, the mean size of the short axis of the metastatic lymph nodes (4.3 ± 3.1 mm) was significantly larger than that of the non-metastatic lymph nodes (3.0 ± 2.0 mm, $p < .001$).

As imaging modalities can detect lymph nodes with a diameter of more than 10 mm of the short axis, we compared the size of the largest lymph node from each case. The mean size of the largest lymph nodes measured along the long axis from 200 individual cases was 14.7 ± 5.8 mm. The mean size of the largest lymph node from 124 patients with nodal metastasis (15.6 ± 5.7 mm) was significantly larger than that from 76 patients without metastasis (13.3 ± 5.8 mm, $p = .009$). There were 595 lymph nodes with a diameter greater 10 mm, 102 of which exhibited metastatic foci (17.1%).

Preoperative estimation of lymph node metastasis by imaging modalities based on each case

Preoperative radiologic estimation of lymph node metastasis by preoperative CT or MRI imaging was compared with metastatic status by histopathologic evaluation in 200 cases. The sensitivity and specificity of preoperative radiologic estimation of metastasis was 56.5% (70/124 cases) and 89.5% (68/76 cases), respectively. The positive predictive value (PPV) and negative predictive value (NPV) of the estimation of lymph node metastasis was 89.7% (70/78 cases) and 55.7% (68/122 cases), respectively, based on preoperative radiologic imaging.

Sensitivity, specificity, PPV, NPV, and overall accuracy of nodal metastasis with different cutoffs based on the number of examined lymph nodes

We then compared the metastatic status of lymph nodes at six different cutoff points, including 6, 8, 10, 12, 15, and 20 mm, based on lymph node size measured along the long axis after fixation. The sensitivity, specificity, PPV, and NPV at each cutoff

Table 2. The sensitivity, specificity, PPV, NPV, and accuracy of six different cutoff points for detecting metastasis by the long axis size of lymph nodes from 200 PDAC cases based on the number of examined lymph nodes

Cutoff point (mm)	Sensitivity	Specificity	PPV	NPV	Accuracy
6	200/412 (48.5)	2,780/4,113 (67.6)	200/1,533 (13.0)	2,780/2,992 (92.9)	2,980/4,525 (65.9)
8	140/412 (34.0)	3,332/4,113 (81.0)	140/921 (15.2)	3,332/3,604 (92.5)	3,472/4,525 (76.7)
10	102/412 (24.8)	3,620/4,113 (88.0)	102/595 (17.1)	3,620/3,930 (92.1)	3,722/4,525 (82.3)
12	62/412 (15.0)	3,809/4,113 (92.6)	62/366 (16.9)	3,809/4,159 (91.6)	3,871/4,525 (85.5)
15	27/412 (6.6)	3,952/4,113 (96.1)	27/188 (14.4)	3,952/4,337 (91.1)	3,979/4,525 (87.9)
20	13/412 (3.2)	4,075/4,113 (99.1)	13/51 (25.5)	4,075/4,474 (91.1)	4,088/4,525 (90.3)

Values are presented as number (%).

PPV, positive predictive value; NPV, negative predictive value; PDAC, pancreatic ductal adenocarcinoma.

point for all 4,525 evaluated lymph nodes are summarized in Table 2. At the cutoff point of 10 mm, the sensitivity was 24.8% (102/412 lymph nodes), specificity was 88.0% (3,620/4,113), PPV was 17.1% (102/595), NPV was 92.1% (3,620/3,930), and overall accuracy was 82.3% (3,722/4,525).

We also compared the metastatic status of lymph nodes at six different cutoff points, including 4, 6, 8, 10, 12, and 14 mm, based on lymph nodes measured along the short axis after fixation. The sensitivity, specificity, PPV, and NPV at each cutoff point for all 4,525 evaluated lymph nodes are summarized in Table 3. At the cutoff point of 10 mm, the sensitivity was 7.0% (29/412 lymph nodes), specificity was 99.0% (4,070/4,113), PPV was 40.3% (29/72), NPV was 91.4% (4,070/4,453), and overall accuracy was 90.6% (4,099/4,525).

Predicted probability of lymph node metastasis based on logistic regression and analysis of ROC

The predicted probability of node metastasis according to lymph node size was assessed by logistic regression analysis. As lymph node size measured along the long axis increased by 1 mm, the odds of node metastasis increased by 1.09-fold. When the long axis size of lymph nodes was more than 10 mm, the predicted probability of nodal metastasis was 12.5%. Fig. 5 demonstrates the ROC curves of six cutoff points for discriminating metastatic lymph nodes from non-metastatic ones measured along the long axis. The AUC value of the ROC curve at the 10 mm cutoff was the largest (0.603; 95% CI, 0.522 to 0.684).

Similarly, as lymph node size measured along the short axis increased by 1 mm, the odds of node metastasis increased by 1.24-

Table 3. The sensitivity, specificity, PPV, NPV, and accuracy of six different cutoff points for detecting metastasis by the short axis size of lymph nodes from 200 PDAC cases based on the number of examined lymph nodes

Cutoff point (mm)	Sensitivity	Specificity	PPV	NPV	Accuracy
4	187/412 (45.4)	2,980/4,113 (72.5)	187/1,320 (14.2)	2,980/3,205 (93.0)	3,167/4,525 (70.0)
6	101/412 (24.5)	3,643/4,113 (88.6)	101/571 (17.7)	3,643/3,954 (92.1)	3,744/4,525 (82.7)
8	52/412 (12.6)	3,929/4,113 (95.5)	52/236 (22.0)	3,929/4,289 (91.6)	3,981/4,525 (88.0)
10	29/412 (7.0)	4,070/4,113 (99.0)	29/72 (40.3)	4,070/4,453 (91.4)	4,099/4,525 (90.6)
12	14/412 (3.4)	4,097/4,113 (99.6)	14/30 (46.7)	4,097/4,495 (91.1)	4,111/4,525 (90.9)
14	8/412 (1.9)	4,112/4,113 (99.9)	8/9 (88.9)	4,112/4,516 (91.1)	4,120/4,525 (91.0)

Values are presented as number (%).

PPV, positive predictive value; NPV, negative predictive value; PDAC, pancreatic ductal adenocarcinoma.

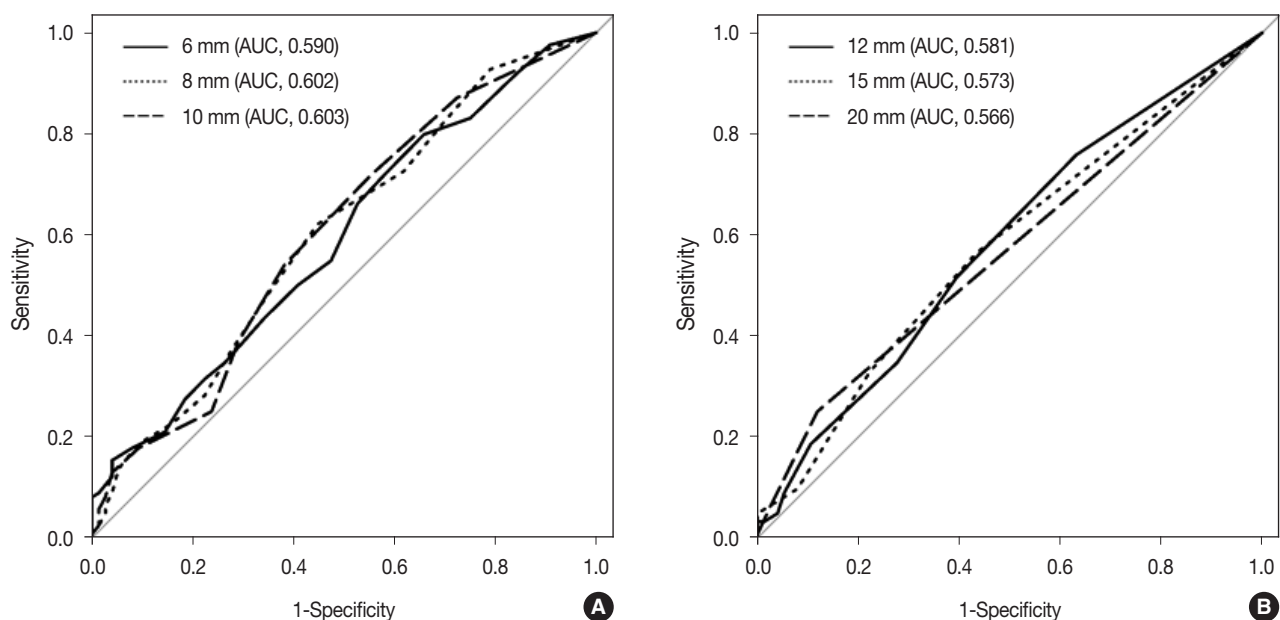


Fig. 5. Receiver operating characteristics (ROC) curves of the estimated lymph node metastasis at six different cutoff points (6, 8, 10, 12, 15, and 20 mm) by measurement along the long axis diameter. (A) ROC curves of estimated lymph node metastasis at cutoff points of 6, 8, and 10 mm. (B) ROC curves of estimated lymph node metastasis at cutoff points of 12, 15, and 20 mm. The largest area under the curve (AUC) value of the ROC curve was 0.603 at the 10 mm cutoff point.

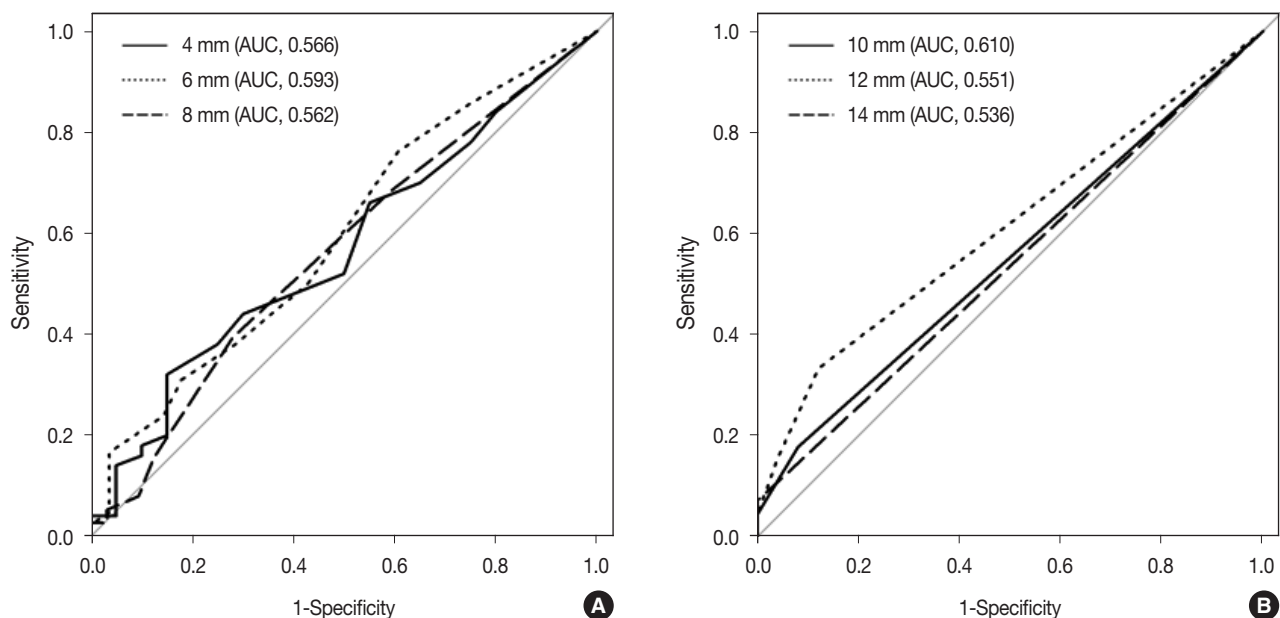


Fig. 6. Receiver operating characteristics (ROC) curves of the estimated lymph node metastasis at 6 different cutoff points of (4, 6, 8, 10, 12, and 14 mm) by measurement along the short axis diameter. (A) ROC curves of estimated lymph node metastasis at cutoff points of 4, 6, and 8 mm. (B) ROC curves of estimated lymph node metastasis at cutoff points of 10, 12, and 14 mm. The largest area under the curve (AUC) value of the ROC curve was 0.610 at the 10 mm cutoff point.

fold. When the short axis size of lymph nodes was more than 10 mm, the predicted probability of nodal metastasis was 32.0%. Fig. 6 demonstrates the ROC curves of six cutoff points for discriminating metastatic lymph nodes from non-metastatic ones measured along the short axis. The AUC value of the ROC curve at the 10 mm cutoff was the largest (0.610; 95% CI, 0.516 to 0.705).

DISCUSSION

Lymph node metastasis is a poor prognostic factor in patients with PDAC [5-8,12]. To better understand the relationship between lymph node size and metastases, we comprehensively evaluated all harvested lymph nodes by measuring the size along the long and short axes and compared the lymph node size and metastasis. The AUC was 0.61 and 0.60 when nodal size was measured along the short and long axis, respectively, at the cutoff points of 10 mm. These results indicate that the performance of preoperative radiologic imaging to predict lymph nodal metastasis is not good.

We observed that when cancer cells metastasized to lymph nodes, the nodal size was significantly larger than that of non-metastatic lymph nodes. However, the mean size difference measured along the long axis between metastatic (6.9 mm) and non-metastatic (5.0 mm) lymph nodes was only 1.9 mm. Similarly,

the mean size difference measured along the short axis between metastatic (4.3 mm) and non-metastatic (3.0 mm) lymph nodes was only 1.3 mm. Traditionally, lymph nodes with a diameter of more than 10 mm measured along the short axis have been radiologically considered as metastatic lymph nodes. When we compared the largest lymph nodes from each case, the mean difference in size measured along the long axis between metastatic (15.6 mm) and non-metastatic (13.3 mm) nodes was only 2.3 mm. When largest lymph nodes from each case were compared, the mean difference in size measured along the short axis between metastatic (8.8 mm) and non-metastatic (7.6 mm) nodes was only 1.2 mm. This difference may not be enough to discriminate metastatic lymph nodes from non-metastatic ones on pre-operative imaging modalities or during surgery.

We evaluated 4,525 lymph nodes and compared node size and metastatic status to determine how to best discriminate between metastatic and non-metastatic lymph nodes. The sensitivity, specificity, PPV, and NPV in the present study was 25%, 88%, 17%, and 92%, respectively, at the 10 mm cutoff point measured along the long axis. The sensitivity, specificity, PPV, and NPV was 7%, 99%, 40%, and 91%, respectively, at the 10 mm cutoff point measured along the short axis. Previous studies compared lymph node size and metastatic status using a small number of lymph nodes. The results of our study and previous reports are summarized in Table 4. The sensitivity and specificity in our

Table 4. Sensitivity, specificity, PPV, NPV, and accuracy of previous studies and the present study

Study	No. of patients	No. of lymph nodes	Measured axis	Cut off point (mm)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Rosch et al. [21]	40	NA	Short	10	36	80	7	42	NA
Palazzo et al. [19]	38	NA	Short	10	19	92	83	34	42
Muller et al. [17]	22	NA	Short	10	20	75	NA	NA	50
Megibow et al. [16]	95	NA	Short	10–15	37	60	47	56	53
Zeman et al. [20]	26	NA	Short	10	NA	NA	NA	NA	58
Midwinter et al. [18]	23	NA	Short	10	33	86	60	67	NA
Roche et al. [15]	9	40	Short	10	14	85	17	8	72.5
Our study	200	4,525	Short and long	10	7	99	40	91	91

PPV, positive predictive value; NPV, negative predictive value; NA, not applicable.

study were comparable with those of previous research (sensitivity, 14%–37%; specificity, 60%–92%) [15–21]. Megibow et al. [16] included large number of examined cases ($n = 95$), and the sensitivity, specificity, PPV, and NPV of their study was 37%, 60%, 47%, and 56%, respectively. The PPV was slightly lower but the NPV was higher in our study, possibly because we included a large number of lymph nodes. Our study had 91% accuracy rate measured along the short axis in estimating nodal metastasis at a 10 mm cutoff point.

In contrast to previous work, we compared the sensitivity, specificity, accuracy, PPV, and NPV of lymph node size to estimate metastasis at several different cutoff points measured along both the long and short axis. Recent technical advances in multi-detector CT scanning allows for reconstructed images at a resolution of 2–5 mm thickness [22]. We were able to best discriminate between metastatic and non-metastatic lymph nodes from ROC analyses with a 10 mm cutoff for the short axis diameter. The PPV of estimating metastatic lymph nodes was 40% based on the ROC analyses.

In our study, the shrinkage effect after FFPE fixation was 6.8%. This suggests that if the long axis of a lymph node is 10 mm after FFPE tissue, this is equivalent to it being 10.7 mm on imaging modalities. The shrinkage effect of lymph nodes (6.8% and 7.9% along the long and short axis, respectively) in the present study was larger than that of kidney and uterine cervix tissues, but smaller than that of other organs in previous studies [24]. The shrinkage effect after FFPE varied between 2.7% to 20%, depending on the organ [24]. One previous study reported that the shrinkage effect of lymph node was 10% when tested with 5% formaldehyde [14].

We measured lymph nodes along the long and short axis regardless of other aspects of metastatic lymph nodes. Some studies measured the short-axis diameter only and used 10 mm as the benchmark to diagnose metastasis [16–21], while other studies used specifically selected cutoff points, including 5 mm, 15 mm,

or 20 mm, for diagnosis. We measured lymph node size the same way as the previous studies by measuring the short-axis diameter and compared size and metastasis at selected cutoff points. In addition, we also measured lymph node size along the long axis, since all pathologic fields use the largest dimension of tumor size. The metastatic lymph nodes were best distinguished from non-metastatic nodes at the cutoff point 10 mm regardless of measuring either long or short axis. However, the performance was not good enough. Therefore, preoperative radiologic evaluation of metastatic lymph nodes may not be dependable.

In summary, metastatic PDAC lymph nodes are larger than non-metastatic ones. Although the metastatic lymph nodes were best distinguished from non-metastatic nodes at the cutoff point 10 mm regardless of measuring either long or short axis, the performance was not good enough. Therefore, nodal size cannot be used a surrogate preoperative marker of lymph node metastasis.

Ethics Statement

Institutional Review Board approval with patient consent waiver was obtained (approval number: 2020-0440).

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Conflicts of Interest

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

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Indirect pathological indicators for cardiac sarcoidosis on endomyocardial biopsy

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Background: The definitive pathologic diagnosis of cardiac sarcoidosis requires observation of a granuloma in the myocardial tissue. It is common, however, to receive a “negative” report for a clinically probable case. We would like to advise pathologists and clinicians on how to interpret “negative” biopsies. **Methods:** Our study samples were 27 endomyocardial biopsies from 25 patients, three cardiac transplantation and an autopsied heart with suspected cardiac sarcoidosis. Pathologic, radiologic, and clinical features were compared. **Results:** The presence of micro-granulomas or increased histiocytic infiltration was always (6/6 or 100%) associated with fatty infiltration and confluent fibrosis, and they showed radiological features of sarcoidosis. Three of five cases (60%) with fatty change and confluent fibrosis were probable for cardiac sarcoidosis on radiology. When either confluent fibrosis or fatty change was present, one-third (3/9) were radiologically probable for cardiac sarcoidosis. We interpreted cases with micro-granuloma as positive for cardiac sarcoidosis (five of 25, 20%). Cases with both confluent fibrosis and fatty change were interpreted as probable for cardiac sarcoidosis (seven of 25, 28%). Another 13 cases, including eight cases with either confluent fibrosis or fatty change, were interpreted as low probability based on endomyocardial biopsy. **Conclusions:** The presence of micro-granuloma could be an evidence for positive diagnosis of cardiac sarcoidosis. Presence of both confluent fibrosis and fatty change is necessary for probable cardiac sarcoidosis in the absence of granuloma. Either of confluent fibrosis or fatty change may be an indirect pathological evidence but they are interpreted as nonspecific findings.

Key Words: Myocarditis; Arrhythmogenic right ventricular dysplasia; Tachycardia, ventricular; Sarcoidosis; Cardiac muscle

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Cardiac sarcoidosis is a myocardial inflammatory disease with non-caseating granulomas [1]. Cardiac sarcoidosis may be cardiac involvement of systemic sarcoidosis, although the heart may be the only organ involved. In contrast to pulmonary sarcoidosis, cardiac involvement of sarcoidosis is often associated with fatal outcome because of ventricular arrhythmia and ventricular dysfunction [2,3]. Clinical studies reveal cardiac involvement in 5%–10% of systemic sarcoidosis cases [4] but others suggest this involvement to be very rare (0.7%) [5] or very common (40%) based on symptomology [6]. The incidence in autopsy series was 20% in a 1952 study [7], but recent studies have demonstrated

variable incidences of 27% [8], 76% in Caucasian patients [4], and 80% in Japanese patients [9]. This variability in incidence may be real; partly due to racial differences in prevalence and due to the development of diagnostic techniques such as the endobronchial ultrasound or cardiac positron emission tomography (PET) [10]. But it may also be related to the variable application of the current diagnostic criteria [1,11].

With respect to the pathologic diagnosis of cardiac involvement in systemic sarcoidosis, “the presence of non-caseating granuloma on histological examination of myocardial tissue with no alternative cause identified” is the current consensus by the Heart

Rhythm Society (HRS) [12]. Although the statement by the HRS is the most reliable description on cardiac involvement in systemic sarcoidosis [2,3,13], its use for pathological diagnosis is limited. Positive diagnosis on endomyocardial biopsy is the only way to confirm cardiac involvement in the current system. The clinical diagnosis of “probable involvement” requires histologic confirmation of extracardiac sarcoidosis. The Japanese Guidelines for Diagnosis of Cardiac Sarcoidosis based on the Study Report on Diffuse Pulmonary Diseases by the Japanese Ministry of Health is more practical for clinical decisions, and the presence of “interstitial fibrosis or monocyte infiltrate over moderate grade” was included as additional minor criteria [10,14]. Based on the current statement, we can only confirm the diagnosis of cardiac sarcoidosis when a granuloma is observed.

Our current pathologic diagnostic practice is somewhat different from the clinical guidelines [12,14] and reported autopsy experiences [4,7-9] because we seldom find definitive granulomas in endomyocardial biopsies of those with clinical cardiac sarcoidosis. Several measures are undertaken to increase the possibility of detecting granuloma; robot-guided biopsy [15], electrocardiogram guided biopsy [16], electro-anatomical mapping system and intracardiac echography guided biopsies [17] are examples. What would be the role of endomyocardial biopsy when we can't find granulomas despite these efforts, but the clinical findings are compatible with cardiac sarcoidosis? There are reports of clinical cardiac sarcoidosis associated with biopsy-confirmed systemic sarcoidosis, but without granuloma in the autopsied heart [18]. Therefore, there is a dilemma for pathologic diagnosis of sarcoidosis in resected whole hearts when no classical granulomas are observed.

We hypothesized that the current pathologic criteria for cardiac sarcoidosis by endomyocardial biopsies are not sensitive enough to detect clinical cases or late phase of possible cardiac sarcoidosis. We therefore claim that pathologic findings other than granuloma may have some significance for clinical decisions and the management of sarcoidosis patients. We therefore categorized findings on endomyocardial biopsies according to the combination of the presence of four pathologic indicators: micro-granulomas, histiocytic infiltration, confluent fibrosis, and fatty change.

We studied 27 endomyocardial biopsies from 25 patients with suspected clinical cardiac sarcoidosis. We also studied four whole hearts: three resected for cardiac transplantation and one examined by autopsy. Clinical, radiological, and therapeutic responses were compared among these pathologic interpretation groups. Our hope is that this categorization approach will correlate with clinical findings, and that these pathologic findings can provide physicians with more information than the presence or absence

of granuloma.

MATERIALS AND METHODS

We reviewed 27 endomyocardial biopsies and three whole hearts after cardiac transplantation from 25 patients at Seoul National University Hospital from 2012 to May 2017. Cases were collected from those with a clinical diagnosis of cardiac sarcoidosis when endomyocardial biopsy was performed. Clinical suspicion of cardiac sarcoidosis was made when there is an arrhythmia and extracardiac sarcoidosis; or certain types of arrhythmia (the second degree Mobitz type II or the third-degree atrioventricular block with or without radiologic features suggestive of cardiac sarcoidosis). We included consecutive cases with different levels of probability suspected by the physician. Clinical summary of 27 biopsies from 25 patients are described in Table 1.

Interpretation criteria of magnetic resonance imaging

The magnetic resonance imaging (MRI) findings were categorized as follows: (1) probable cases: these cases involve multiple patchy enhancement at the subepicardial or mid-myocardial layers, which do not correspond to the expected territories of coronary arterial supply. Involvement of the basal part of the interventricular septum, irregular thinning of the wall, and ejection fraction less than 50% are considered probable cases; (2) possible cases: these cases have features similar to those of probable cases, but diseases other than cardiac sarcoidosis could be considered a cause of the lesion; (3) nonspecific findings: these cases have minor and focal lesions of fibrosis, which may be interpreted as early lesions of sarcoidosis or other conditions; and (4) unlikely: these cases have radiological features more likely to indicate an ischemic condition or other specific conditions.

Interpretation criteria of four pathologic findings

The endomyocardial biopsies were stained with hematoxylin and eosin stain, Masson's trichrome (MT) stain, and immunohistochemical stain for CD68. The pathological findings were analyzed based on four morphological changes: micro-granulomas, increased histiocytes, confluent fibrosis, and fatty tissue. Subsequently, cases were described and analyzed according to groups of morphologic findings.

Micro-granuloma

A granuloma is defined as a collection of histiocytes. The size of granulomas vary and there is no consensus on the minimum size of granuloma, although it is generally accepted that the gran-

Table 1. Summary of information on cases of clinically suspected cardiac sarcoidosis

Case No. ^a	Sex	Age ^b	Extracardiac sarcoidosis	Steroid effect	Cardiac rhythm	Heart block ^c	Ventricular arrhythmia ^d	LVEF (%)	Heart TPL	MRI diagnosis
Group 1										
1-1	M	>40	No	No	Sinus	No	No	22	Yes	Probable
1-2	M	>40	No	No	Sinus	No	No	33	Yes	Probable
1-3	F	>50	Not definite	No	Sinus	No	No	51	No	Probable
1-4	M	>60	No	Unknown	Paroxysmal AF	No	No	30	No	×
1-5	F	>60	No	No	Sinus	No	No	42	No	Probable
Group 2										
2-1	F	>70	No	No	AF	No	No	15	No	Probable
2-2	F	>70	No	No	AF	No	No	15	No	Probable
Group 3										
3-1	M	>50	No	No	Sinus	Yes	Yes	44	No	Probable
3-2	F	>70	No	Yes	Paroxysmal AF	Yes	No	40	No	Possible
3-3	M	>40	No	No	Sinus	Yes	Yes	36	Yes	Nonspecific
3-4	F	>90	No	Unknown	Sinus	Yes	Yes	30	No	Probable
3-5	F	>50	Yes (lung, lymph node)	Unknown	Sinus	Yes	Yes	50	No	×
3-6	F	>70	No	Yes	Sinus	No	No	22	No	Probable
Group 4										
4-1	M	>60	No	Unknown	Sinus	No	Yes	30	Yes	Probable
4-2	M	>40	No	No	Sinus	No	No	33	Yes	Probable
4-3	M	>70	No	Unknown	AF	No	No	56	No	Nonspecific
Group 5										
5-1	M	>70	No	No	Sinus	No	No	48	No	Probable
5-2	F	>60	No	Yes	Sinus	No	No	35	No	Nonspecific
5-3	F	>70	No	Yes	Paroxysmal AF	No	Yes	35	No	Nonspecific
5-4	M	>60	No	No	Sinus	No	No	48	No	Nonspecific
5-5	M	>70	Yes (lymph node)	No	Sinus	No	Yes	42	No	Unlikely
5-6	F	>50	Yes (skin)	Yes	Sinus	Yes	No	61	No	Unlikely
Group 6										
6-1	M	>50	No	Unknown	Sinus	Yes	Yes	46	No	×
6-2	M	>30	No	No	Sinus	No	No	30	No	Nonspecific
6-3	M	>20	Yes (lymph node)	Yes	Sinus	No	No	25	No	Nonspecific
6-4	M	>60	No	Unknown	Sinus	No	No	28	No	Nonspecific
6-5	M	>10	No	Unknown	Sinus	No	No	54	No	Unlikely

Group 1, endomyocardial biopsies with micro-granuloma as well as histiocytic infiltration, confluent fibrosis and fatty change; Group 2, endomyocardial biopsies with histiocytic infiltration, confluent fibrosis and fatty change but without micro-granuloma; Group 3, endomyocardial biopsies with confluent fibrosis associated with fatty tissue infiltration; Group 4, presence of confluent fibrosis without associated fatty tissue; Group 5, presence of fatty tissue without associated fibrosis; Group 6, none of four possible indicators on endomyocardial biopsy.

LVEF, left ventricular ejection fraction; TPL, transplantation; MRI, magnetic resonance imaging; M, male; F, female; AF, atrial fibrillation.

^aCases 1-2 and 4-2 are a same case, cases 2-1 and 2-2 are another same case; ^bAge is expressed in 10-year interval; ^cSecond degree Mobitz type II or third-degree atrioventricular block; ^dSustained ventricular tachycardia or ventricular fibrillation.

uloma has a size more than 30 or 50 histiocytes. In this study, we interpreted a small nodular collection of 5–10 histiocytes as a micro-granuloma. CD68 staining was necessary to find a micro-granuloma (Fig. 1).

Increased histiocytes

In some cases, infiltration of histiocytes was observed at the myocardial interstitium. We classified a significant increase in histiocytes in the interstitium when histiocytes are at least three times more than their usual frequency. CD68 immunostaining

was necessary to count the number of histiocytes.

Confluent fibrosis

Confluent fibrosis replacing more than 30–50 myocardial cells was considered as a significant post-necrotic lesion in this study. The fibrotic lesion was more or less edematous and was associated with some inflammatory cells. We did not count the slender collagen fibers in the interstitium without myocyte damage, which was commonly encountered as interstitial fibrosis. Multifocal spotty fibrosis following necrosis of individual myocardial cells

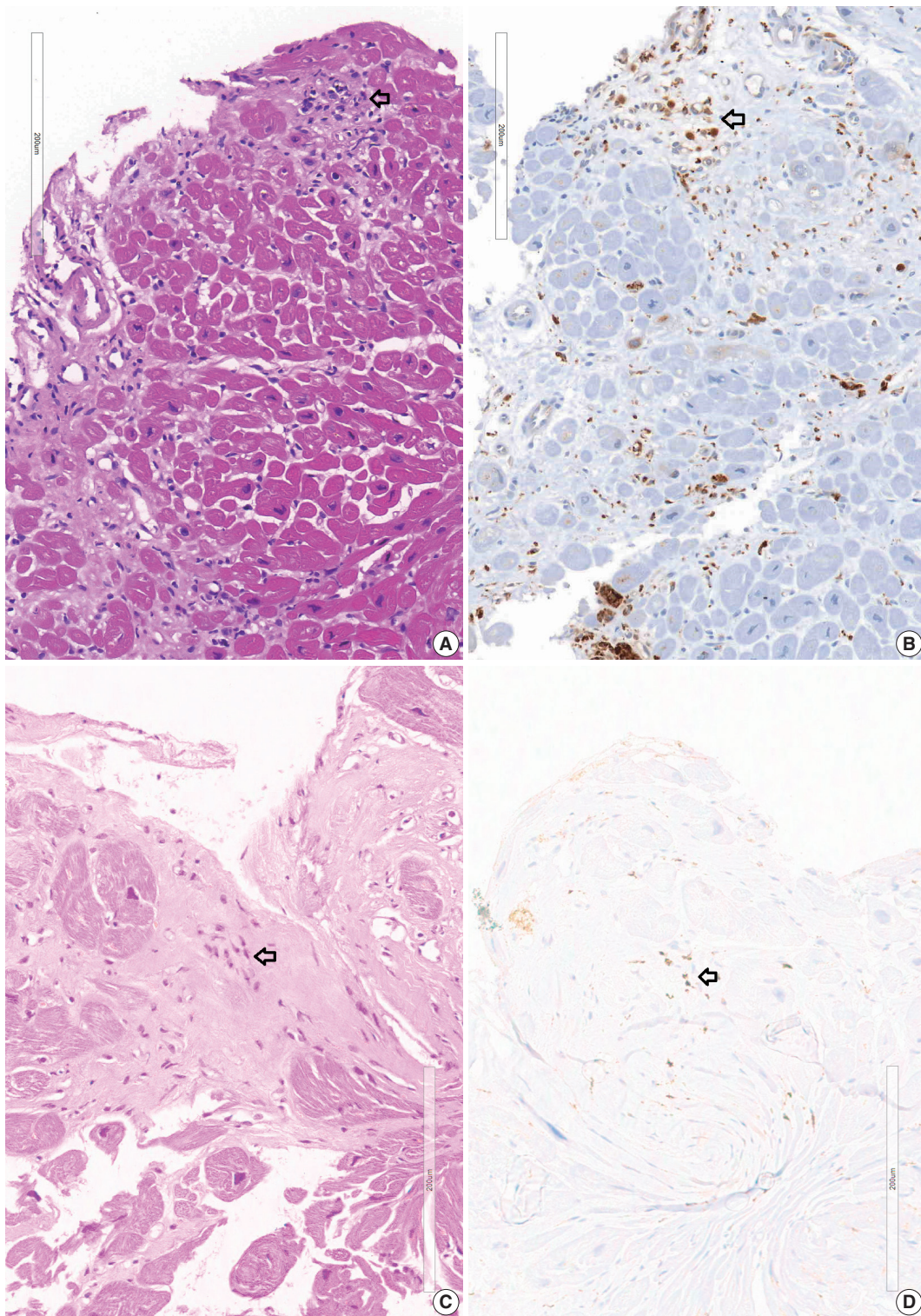


Fig. 1. Micro-granuloma on the endomyocardial biopsy. (A) Endomyocardial biopsy at 2 years prior to the transplantation of case 1-1 shows confluent fibrosis with edematous stroma. Three foci of infiltration of histiocytes and lymphocytes (arrow) are seen at the margin of fibrosis which is the interface between the fibrosis and myocardium. (B) CD68 staining of the same specimen showing histiocytic infiltration at the micro-granulomas (arrow). (C) Endomyocardial biopsy of case 1-3 shows a micro-granuloma (arrow) of 15 cells in the fibrotic zone. (D) CD68 immunostaining of endomyocardial biopsy of case 1-3 shows positive staining (arrow) on histiocytic marker.

(replacement fibrosis) was also excluded in this study. MT stain was useful to reveal confluent fibrosis.

Fatty change

Post-inflammatory infiltrated fat cells were also recognized as individual fat cells in the fibrotic myocardial scar. Subendocardial accumulation of fat cells was also observed. Sometimes young fat cells with small and bubbly cytoplasm were noted. Differentiating them from fatty tissue as a normal component of the interstitium between myocardial bundles and perivascular space or as an extension of epicardial fatty tissue was difficult. Therefore, we noted infiltrations of fatty tissue of more than 10 adipocytes, which were interpreted as the presence of fatty tissue in this study.

Four cases received cardiac transplantation and three transplant hearts were examined by conventional gross dissection of the heart. A semi-quantitative histological mapping was performed on a sectional plane of a heart. Pathologic features were examined to determine granulomas and other related pathologic findings. An autopsied heart was examined, and microscopic images were added as supplementary material to compare classical sarcoidosis before treatment with our clinical cases in endomyocardial biopsies and heart transplantation.

RESULTS

Histopathologic findings of 27 endomyocardial biopsies

Endomyocardial biopsies with micro-granuloma as well as histiocytic infiltration, confluent fibrosis, and fatty change

Five endomyocardial biopsies were included in group 1 (Table 1). None of the five endomyocardial biopsies were interpreted as granuloma in the initial pathologic report because of small atypical granuloma-like collections of 5–10 histiocytes. We interpreted these cases as micro-granulomas based on the current definition for this study. One case was reported as myocarditis and four others had confluent fibrosis associated with increased histiocytes. One case was the second endomyocardial biopsy after the first biopsy showed no micro-granulomas two years previously. No cases had any evidence of extracardiac sarcoidosis. All five cases showed confluent fibrosis, increased histiocytes, and fatty change (Fig. 1). Two cases received cardiac transplantation after which the resected hearts showed granulomas (Fig. 2).

Four of these patients had MRI findings indicating probable sarcoidosis. One case was not checked for cardiac MRI but clinical findings were suggestive of cardiac sarcoidosis; global hypo-

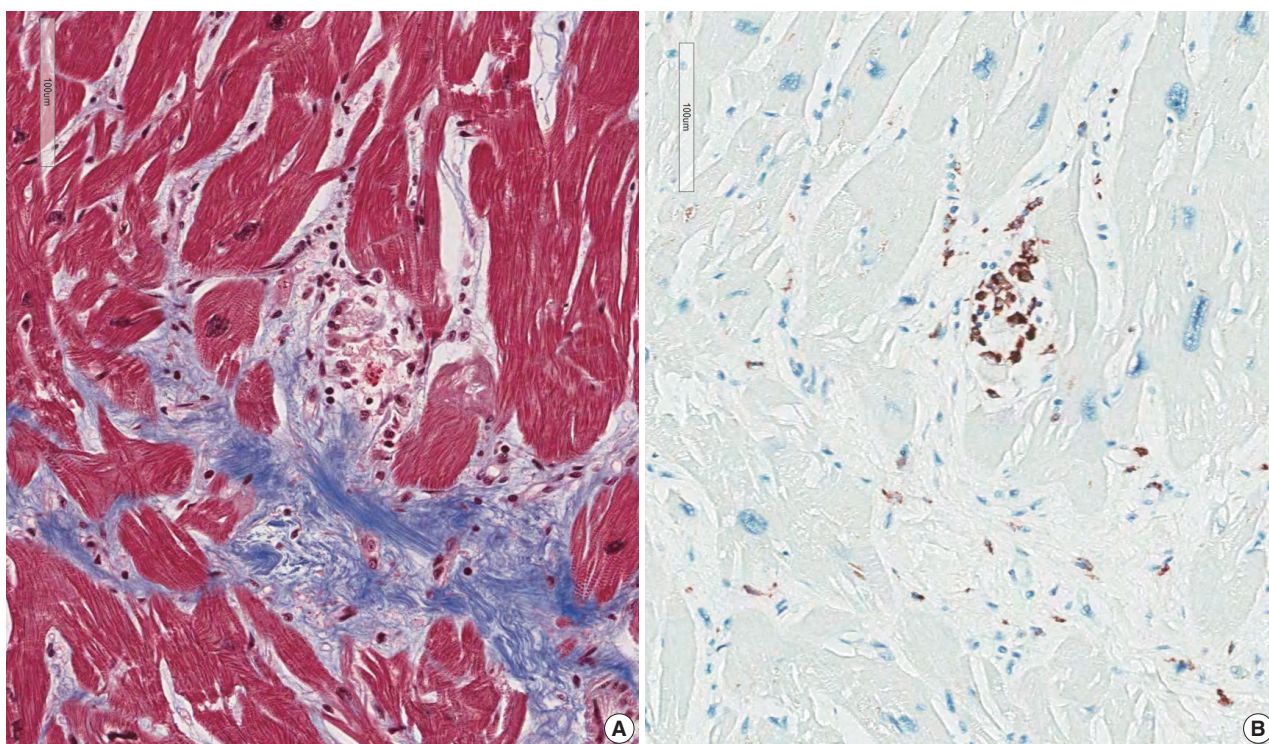


Fig. 2. Micro-granuloma in an explant heart. (A) Magnification of the ventricular myocardium of explant heart in case 1-2 reveals interstitial fibrosis and a few small granulomas. (B) Immunohistochemical staining on CD68 in the same area shows a small nodular collection of histiocytes or zone of scattered histiocytes.

kinesia on the echocardiography and low left ventricular ejection fraction (30%) without any evidence of ischemic heart disease.

Endomyocardial biopsies with histiocytic infiltration, confluent fibrosis, and fatty change but without micro-granuloma

Two endomyocardial biopsies from a patient (group 2) showed an increase in histiocytes, confluent fibrosis, and fatty change. Two endomyocardial biopsies showed confluent fibrosis admixed with fatty change and increased histiocytes in the same area within the biopsied tissue (Fig. 3). Granuloma was not definitive but nodules of 3–5 histiocytes were scattered. MRI findings were compatible with sarcoidosis, although extracardiac evidence of sarcoidosis was lacking.

Endomyocardial biopsies with confluent fibrosis associated with fatty tissue infiltration

Confluent fibrosis was associated with fatty infiltration in six cases (group 3). Fatty change was observed at the immediate sub-endocardium or in the fibrous scar. Adipocytes showed variable sizes and were admixed with fibrosis, particularly at the margin of the fatty tissue and at the junction of the myocardium (Fig. 4).

One case was associated with extracardiac sarcoidosis in the lungs and mediastinal lymph nodes. MRI findings of four cases were compatible with sarcoidosis, though one case was excluded. MRI was not performed in two cases. The echocardiographic results of two cases showed unexplained ventricular dysfunction (left ventricular ejection fraction of 33%) with global hypokinesia and basal septal akinesia with thinning, respectively, which suggested cardiac sarcoidosis.

Presence of confluent fibrosis without associated fatty tissue

Three cases in group 4 showed confluent fibrosis (Fig. 5). Two cases showed hyalinized fibrosis, and one case showed fibrosis and edematous stroma. Confluent fibrosis is not evident in a small biopsy, but fibrosis larger than five times the diameter of myocardial cells can be interpreted as a scar related to a previous site of granuloma. Perivascular interstitium sometimes expanded to mimic confluent fibrosis and a fibrotic lesion around the blood vessels should be excluded before interpreting post-granuloma scar. Confluent fibrosis may be young edematous lesion, but may also be a hyalinized scar.

Presence of fatty tissue without associated fibrosis

Six cases in group 5 showed fatty tissue without associated fibrosis. Fatty tissue was an expansion of perivascular fatty tissue

or fatty tissue between myocardial muscle groups (Fig. 4D).

One case showed MRI findings consistent with sarcoidosis, whereas three cases showed MRI findings not consistent with sarcoidosis. Two other cases had sarcoidosis in the mediastinal lymph node or skin, but the MRI findings did not support sarcoidosis.

None of four possible indicators on endomyocardial biopsy

Six cases showed normal myocardium or only minimal interstitial fibrosis (group 6). Three cases showed nonspecific findings on MRI. Two cases showed unlikely diagnosis on MRI. One case did not have MRI results, but the echocardiography showed normal left ventricular function with unexplained basal septal akinesia with thinning, because of which cardiac sarcoidosis was suspected.

Pathology of three cases of cardiac transplantation and an autopsy case

One case was clinically diagnosed as cardiac sarcoidosis with dilated right ventricular chamber. The initial pathological diagnosis of endomyocardial biopsy in this > 40 years old man was myocarditis possibly related to systemic lupus erythematosus. He was treated with cyclophosphamide, methylon, cyclosporine A, and azathioprine. MRI findings were compatible with sarcoidosis (Supplementary Fig. S1).

Review of endomyocardial biopsy revealed three foci of a very small collection of 8–10 histiocytes, which was not interpreted as a granuloma at the initial pathologic examination (Fig. 1A).

The resected heart weighed 350 g. There were multiple white scars on the myocardium (Fig. 6). The right ventricular free wall was severely fibrotic and no myocardial tissue was seen at some parts of the right ventricular wall. The trabeculae at the right ventricular apex were severely atrophic and only fibrous cords were noted (Supplementary Fig. S2). An entire plane of the short-axis of the heart showed large scars scattered at the left and right ventricles and ventricular septum (Fig. 6B). The distribution of the scars was comparable to that of multifocal subendocardial enhancement by cardiac MRI (Supplementary Fig. S1). In the multiple myocardial scars, scattered histiocytes and a few micro-granulomas were found (Supplementary Fig. S2).

The second case had hypertrophied right and left ventricles with multifocal fibrosis.

The initial clinical diagnosis of this > 40 years old man was cardiac amyloidosis. Echocardiography showed a borderline increase in the left ventricular wall thickness with apical regional wall motion abnormalities. Cardiac MRI showed patchy, irregular, and delayed enhancement sparing subendocardium sug-

gesting non-ischemic cardiomyopathy (Supplementary Fig. S3). PET with 2-deoxy-2-[^{18}F]fluoro- D-glucose (FDG-PET) did not show hot uptake in the myocardium. Holter monitoring showed non-sustained ventricular tachycardia of three beats on two occasions.

Endomyocardial biopsy was performed twice at ages 49 and 51. One biopsy at age 51 showed focal patchy fibrosis with a single micro-granuloma (Supplementary Fig. S4).

The resected heart after transplantation at age 51 weighed 560 g. Both right and left ventricular walls were hypertrophied.

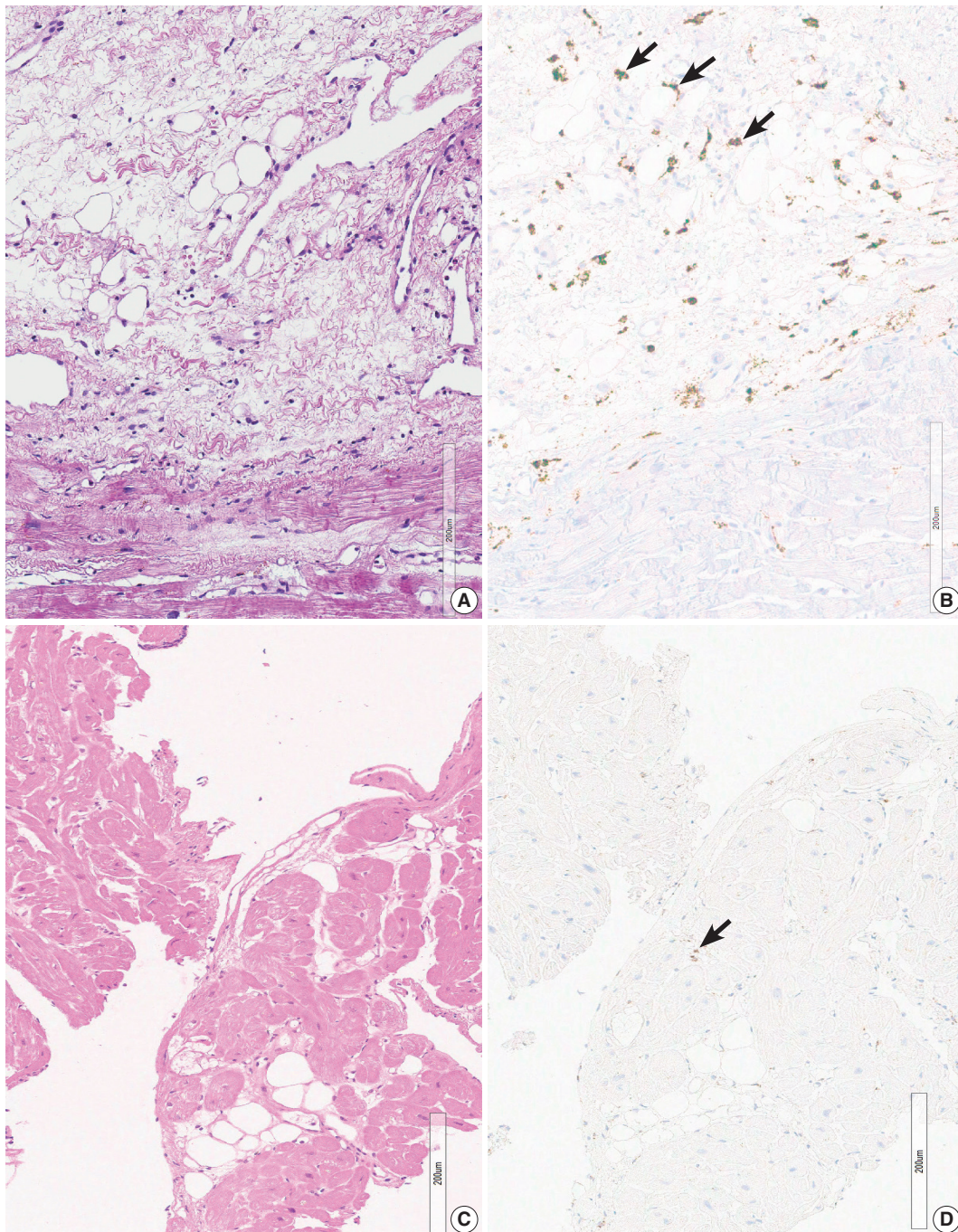


Fig. 3. Hystiocytic infiltration, confluent fibrosis and fatty change. (A) Confluent fibrosis, associated fatty change within the fibrosis in case 2-1. (B) CD68 staining in the same area shows increased histiocytes (arrows) scattered in the fibrous area. (C) Confluent fibrosis, associated fatty change within the fibrosis in case 3-1. (D) CD68 staining in the same area shows very rare or no increase of histiocytes (arrow) in the fibrous area.

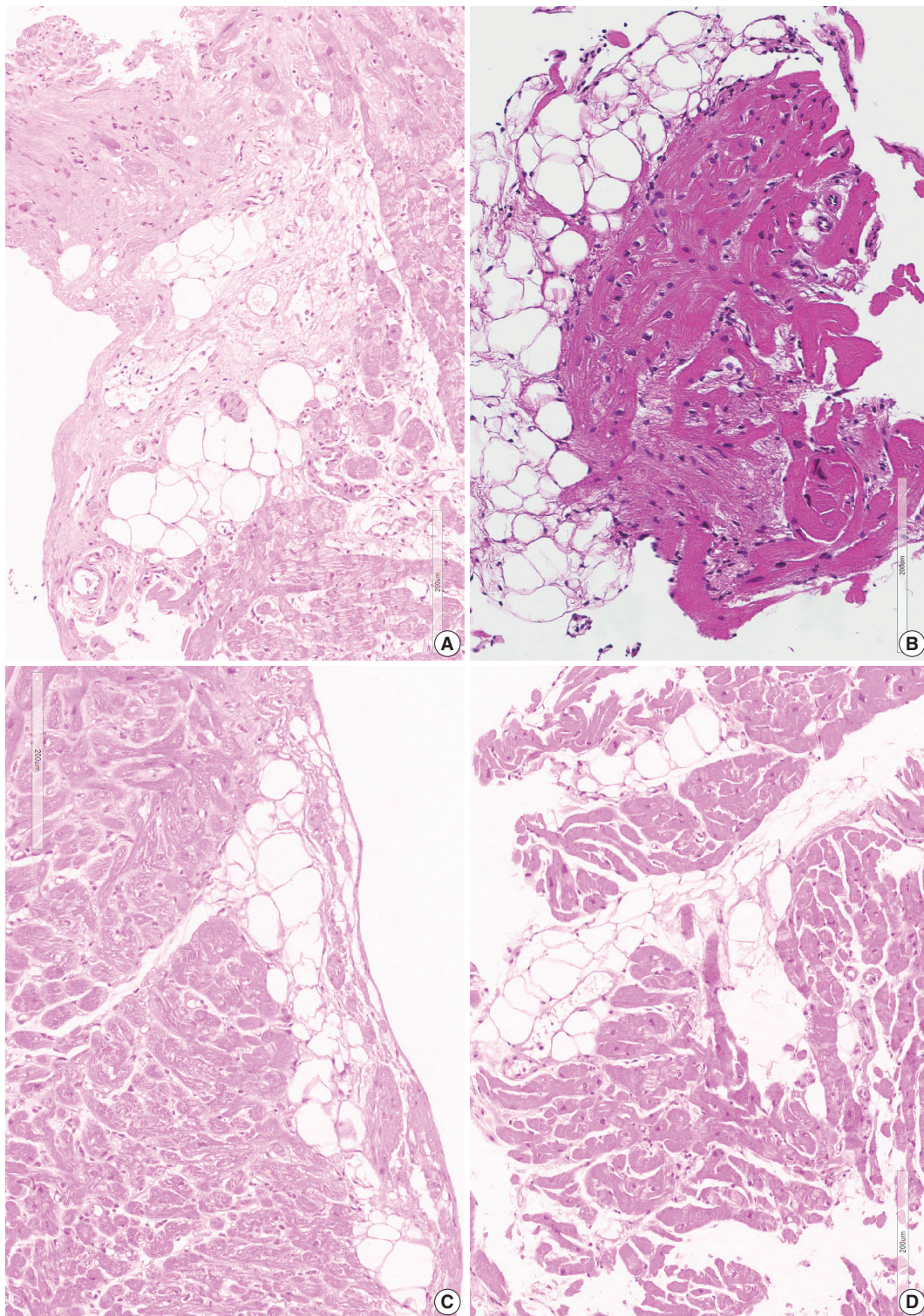


Fig. 4. Different types of fatty changes in endomyocardial biopsies. (A) Fatty infiltration in the background of confluent fibrosis (case 3-2). (B) Fatty tissue with variable sizes of adipocytes and adjacent myocardium also show post-inflammatory fibrosis (case 1-5). (C) Subendocardial deposition of fatty tissue. Slender fibrotic zone is visible at the margin of fatty area (case 3-5). (D) Fatty infiltration between the myocardial bundles. Adjacent myocardium is normal without fibrosis or inflammation (case 5-4).

There were multiple white scars in the myocardium, particularly in the left ventricle, the ventricular septum, and papillary muscles (Fig. 7). Histologically, the scars were dense fibrous scars and

had fatty replacement. At least five micro-granulomas and histiocytes were scattered (Fig. 2).

The third case was clinically suspected as cardiac sarcoidosis

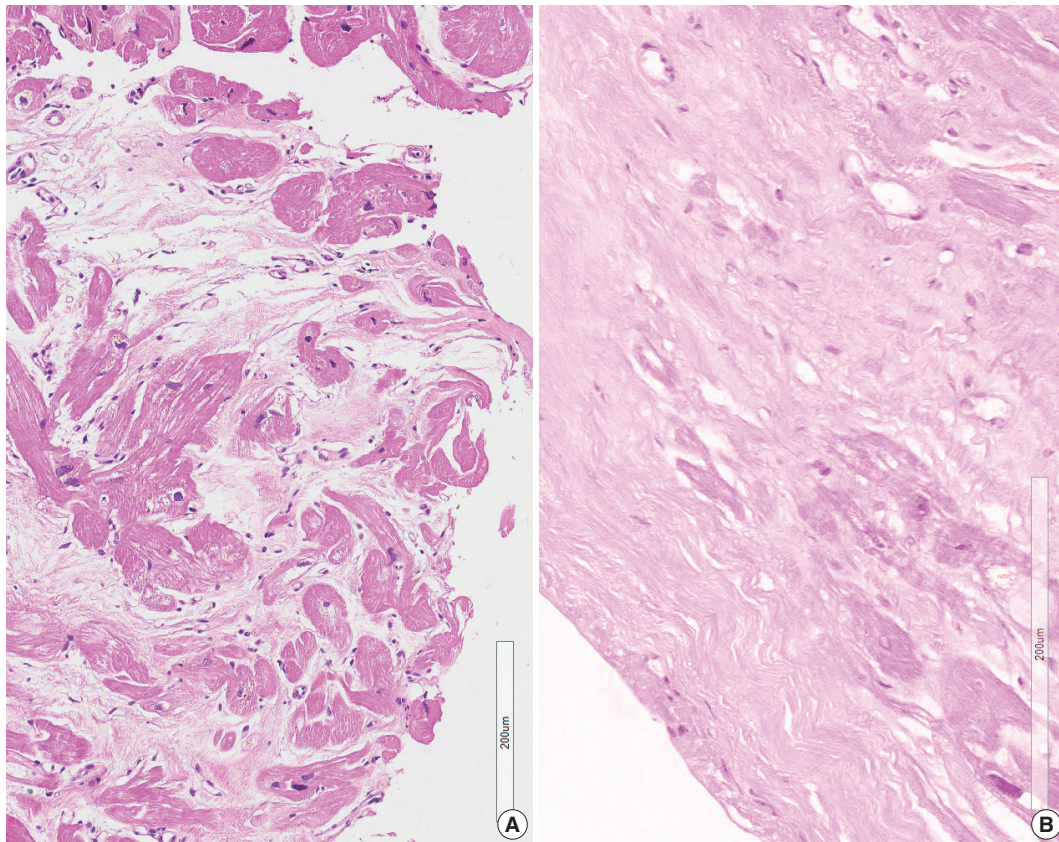


Fig. 5. Confluent fibrosis without associated fatty tissue. (A) Confluent fibrosis is not evident in a small biopsy but fibrosis bigger than five times the diameter of the myocardial cells was interpreted as a scar related to a granuloma (case 4-2). (B) Broad scar at the endocardial zone. Some adipocyte-like spaces were found but the scattered individual spaces were not interpreted as fatty change (Case 4-3).

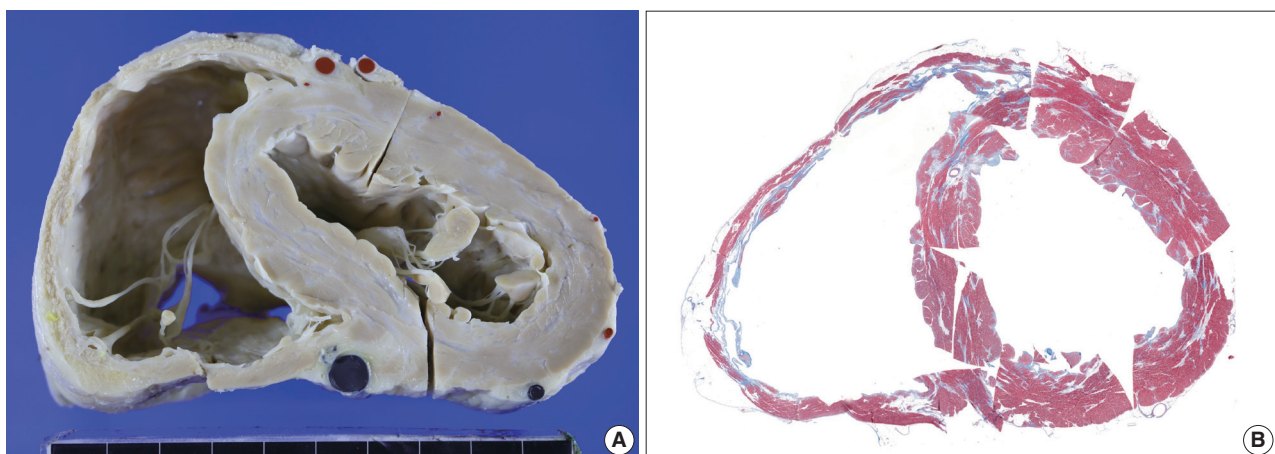


Fig. 6. A short-axis sectional view of the first transplant heart with sarcoidosis and fibrosis. (A) A short-axis sectional view of the heart shows multifocal confluent fibrosis involving both ventricles. The right ventricular thinning and dilatation are prominent. Coronary arteries and cardiac veins are filled with red and blue silicone rubber cast. (B) Histotopographic mapping of a short-axis plane of the heart by Masson's trichrome staining reveals prominent fibrosis (in blue color) in the right ventricular free wall and patchy fibrosis in the ventricular septum and left ventricle.

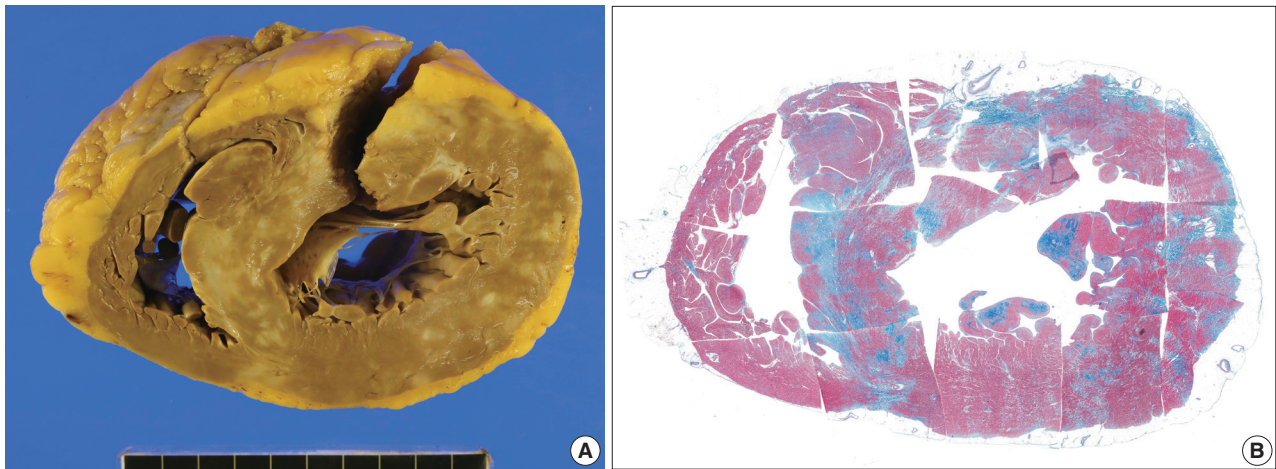


Fig. 7. The second transplant heart with sarcoidosis and hypertrophied ventricles. (A) Sectional view of the heart shows multifocal confluent fibrosis involving predominantly left ventricle and the ventricular septum. Epicardial fatty tissue is prominent in the right ventricle but the myocardium is not much involved. (B) Histotopographic mapping of a short-axis plane of the heart by Masson's trichrome staining reveals prominent fibrosis (in blue color) in the interventricular septum and the left ventricular free wall. Distribution of the fibrosis is prominent but not limited to the subepicardial zone.

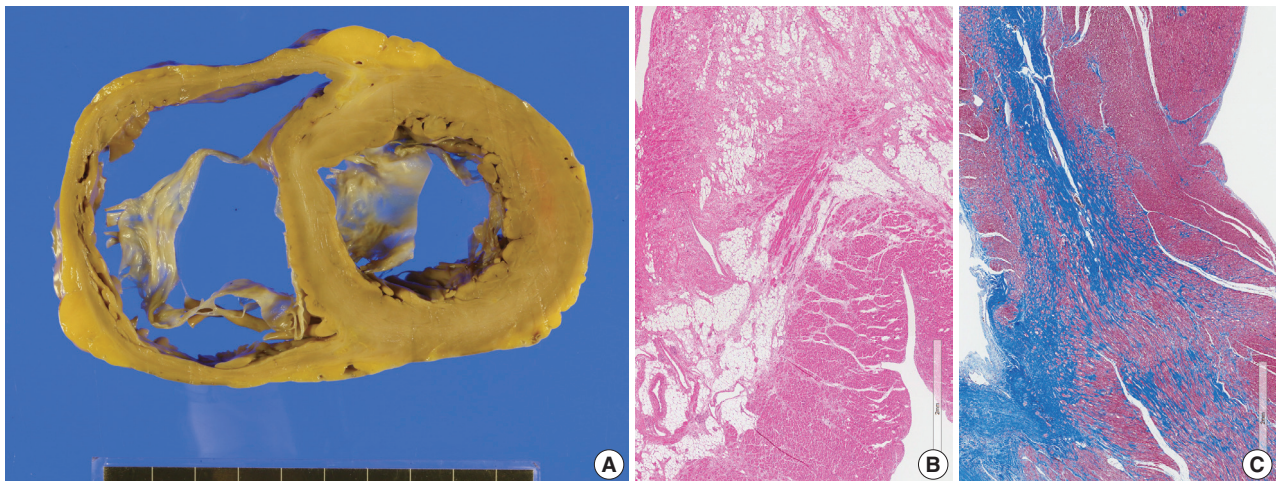


Fig. 8. The third transplant heart mimicking sarcoidosis on microscopy but not likely of sarcoidosis on macroscopic view. (A) The left ventricle is dilated and hypertrophied. Multifocal and diffuse fibrosis was noted on gross examination. Fatty infiltration was prominent at the anterior part of the ventricular septum and mid-septal fibrosis was evident. (B) Fatty change associated with myocardial fibrosis was seen at the anterior part of the ventricular septum. (C) Low magnification of the mid-septal fibrosis (Masson's trichrome stain).

but pathological study denied the diagnosis after examination of the resected heart. The initial clinical presentation of a > 40 years old man was dyspnea on exertion and edema in the lower extremities. Complete atrioventricular block was noted, and a permanent pacemaker was inserted. Heart transplantation was performed at age 45. MRI was not performed, but ^{18}F -FDG-PET revealed a localized hypermetabolic lesion at the apex and apical and mid-inferior antero-septal wall. Computed tomography angiography showed no significant myocardial fibrosis.

The endomyocardial biopsy revealed three large pieces with different features. One piece showed confluent fibrosis and focal

fatty infiltration. The second and third pieces were relatively well-preserved myocardium with interstitial fibrosis.

The resected heart weighed 365 g and both ventricles were enlarged. There was multifocal fibrosis in the left ventricular wall and septum. The mid-septal fibrosis was prominent and was diffuse rather than patchy. Fatty infiltration was seen at the anterior end of the basal part of the ventricular septum (Fig. 8). The fibrosis pattern was diffuse in the myocardium, but dense hyalinized fibrosis was seen in the mid-septal area (Fig. 8). We interpreted this explant heart as non-specified dilated cardiomyopathy rather than sarcoidosis.

An autopsy case (a man who died suddenly and a heart with many classical granulomas)

We reviewed a previously reported autopsied heart with sarcoidosis [19]. Macroscopic findings of the classical cardiac sarcoid lesion and histologic details are enclosed as supplementary materials. This 43-year-old man presented with syncope without any significant clinical history. Forensic autopsy revealed enlarged lymph nodes with sarcoid granuloma. The heart weighed 490 g and there were several conglomerated mass-like lesions (5.0×1.2 cm) at the anterior wall of the left ventricle adjacent to the left anterior descending coronary artery (Supplementary Fig. S5). Histological examination showed active non-caseating granulomata involving multiple sites in the left and right ventricles. Lesions were subepicardial, subendocardial, or transmural in the ventricular wall (Supplementary Fig. S6). Fatty infiltration or fatty replacement was also noted even in the deep myocardium. Immunohistochemistry revealed CD68-positive histiocytes and CD3-positive T-lymphocytes (Supplementary Fig. S7).

DISCUSSION

Presence of classical granuloma in cardiac tissue is considered as the gold standard for diagnosis of sarcoidosis although infectious and immunologic causes are suggested in some cases [20,21]. Pathologists are asked to report the presence or absence of granulomas in small endocardial biopsies, and granulomas are not seen in most biopsies. When we don't see any granulomas, it would be more useful for clinicians whether the case has some indirect or suggestive finding, rather than excluding the case from pathological diagnosis of sarcoidosis. We believe four histological findings and their combinations will have some value for pathological support of clinical practice on patients with cardiac sarcoidosis.

We compared our findings with radiological findings (Table 2). The presence of micro-granulomas (four cases, excluding a case without MRI findings) or increased histiocytic infiltration (two biopsies from one patient) was always (100%) associated with fatty infiltration and confluent fibrosis, and these cases showed radiological features of probable sarcoidosis. Among six cases with fatty change and confluent fibrosis, five cases had MRI findings. The MRI findings of three cases indicated probable cardiac sarcoidosis (3/5 or 60% possibility of sarcoidosis by radiology). When a single finding of either confluent fibrosis or fatty change was present, three of nine cases (33%) radiologically supported sarcoidosis. From these observations, we can categorize patients with two (fatty change and confluent fibrosis) or more indicators as cases with probable sarcoidosis on endomyocardial biopsy.

Clinical findings were so diverse that they matched poorly with pathological or radiological findings. None of the six cases with confirmed granulomas or increased histiocytes showed heart block. Five among 12 cases (42%) with pathological features of more than two indicators (confluent fibrosis and fatty change) had heart block. There were seven cases with heart block and five of them (71%) were probable for cardiac sarcoidosis by our pathological criteria.

In regard to corticosteroid treatment on cardiac sarcoidosis, cases at early inflammatory phase will get more benefit than cases with end-stage fibrotic lesions [22,23]. The detection of active definitive granulomas would be a definitive indication for steroid treatment. It is not surprising therefore that there was a poor correlation with the pathological parameters in this series.

The non-caseating granuloma is the classic histopathology of sarcoidosis [1,24] but there exists a spectrum of histology. Upon review of autopsy cases of cardiac sarcoidosis, granulomatous lesions were found in most of cases (108 patients) but in some cases (5 patients) myocardial scarring was a dominant lesion [18].

Table 2. Numbers of cases in our interpretation categories and radiologic features

Our interpretation categories ^a	MRI finding					Total
	Probable	Possible	Nonspecific	Unlikely	Not checked	
1. Positive for cardiac sarcoidosis: presence of four indicators	4	-	-	-	1	5
2. Probable for cardiac sarcoidosis (1): Presence of three (confluent fibrosis, fatty change and increased histiocytes) but no micro-granuloma	2	-	-	-	-	2
3. Probable for cardiac sarcoidosis (2): Presence of confluent fibrosis and fatty tissue infiltration	3	1	1	-	1	6
4. Nonspecific (1): Confluent fibrosis without associated fatty tissue	2	-	1	-	-	3
5. Nonspecific (2): Fatty tissue without associated fibrosis	1	-	3	2	-	6
6. Nonspecific (3): None of four possible indicators on endomyocardial biopsy	-	-	3	1	1	5
Total	12	1	8	3	3	27

Four presumptive indicators of cardiac sarcoidosis are micro-granuloma, confluent fibrosis, fatty change and increased histiocytes.

MRI, magnetic resonance imaging.

^aDetails of case groups 1–6 are shown in Table 1.

Histologic features were variable and the spectrum was divided into four types: exudative type, granuloma type, combined type, and fibrotic type (Table 3) [25]. Some extreme examples presented as cases without granuloma in any internal organs at autopsy, which had several years history of cardiac arrhythmia, and where the initial histological diagnosis was sarcoidosis in the lymph node [18]. Our cases with very small granuloma-like lesions on endomyocardial biopsy were interpreted as myocarditis or ignored instead of being interpreted as granulomas. Each granuloma was composed of 5–10 cells, and giant cells were not found.

A micro-granuloma is a type of small granuloma or granuloma-like lesion. The micro-granuloma was described in a medical dictionary as “a term of art referring to an aggregate of less than 25 epithelioid histiocytes” [26] and is associated with Crohn’s disease [27]. The micro-granuloma in our new definition was smaller than that of the dictionary. We further suggested the increase in scattered histiocytes as a variant of a lesion with similar significance of granuloma in cardiac sarcoidosis. A recent study revealed that the presence of CD3-, CD68-, and CD163-positive cells in endomyocardial biopsies of patients with dilated cardiomyopathy was associated with cardiac fibrosis and poor clinical outcomes [28]. The increase in CD68-positive cells may be an indicator of active myocardial inflammatory conditions, including sarcoidosis.

It is well documented that the histologic features of sarcoid granuloma are resolved by corticosteroid treatment [29,30] and spontaneous regression was observed in one-third of pulmonary sarcoidosis [31]. The case of cardiac sarcoidosis without granuloma at autopsy was interpreted as a result of steroid treatment in a previous report [18]. As is shown in the variable features of granulomas in the cardiac sarcoidosis on autopsied heart (Supplementary Fig. S7), presence of giant cells and histiocytes varies

in the lesions and they will resolve to fibrotic lesions. Therefore, we would interpret the stromal micro-granuloma-like lesions as a healed stage of collection of scattered histiocytes.

The significance of confluent scars in the myocardium is also debatable. We agree that they are large replacement fibrosis [32] but they are different from the large scars of myocardial infarct by their distribution related to the coronary arterial supply. It is not always possible to obtain a large specimen on endomyocardial biopsy to find confluent fibrosis but it was possible in some cases. Macroscopic classification of myocardial lesions was described in three classes: spotty pattern, conglomerate band-like pattern, and dendritic pattern (Table 3) [25]. The detection of such patchy fibrosis may indicate the pathologic substrates of an apparent conduction delay of the electrical impulse and fractionation due to asynchronous activation in different tracts [32]. We therefore interpret large scars as probable evidence for cardiac sarcoidosis.

The presence of mature fat or “fibro-fatty replacement of myocardium” in endomyocardial biopsies is a hallmark of arrhythmogenic right ventricular cardiomyopathy [33–35]. However, the presence of mature epicardial fat in the endomyocardial biopsy is common. In such samples, we observed completely normal fatty tissue with direct attachment to the normal myocardium (Fig. 4D). Careful observation of fatty tissue in our endomyocardial biopsies revealed some different microscopic features, including fatty tissue associated with fibrosis and histiocytes. These fat cells often had small or variable cytoplasmic contours. We interpreted these as recently transformed fatty changes at the endocardial zone, or deep from the pericardium. It is not easy to differentiate these post-inflammatory fatty changes from those seen in the old myocardial infarcts. Fatty changes alone are not significant, but if fatty change is associated with young scars and histiocytes, it may be a clue to sarcoidosis.

Table 3. Macroscopic and histological classification on the spectrum of pathology in the cardiac sarcoidosis and cardiac fibrosis in the literature

Morphologic patterns
1. Macroscopic classification of myocardial lesions in cardiac sarcoidosis [25]
- Spotty pattern
- Conglomerate band-like pattern
- Dendritic pattern
2. Histologic features of myocardial lesions in cardiac sarcoidosis [25]
- Exudative type: marked lymphocytic infiltration, diffuse edema, collection of histiocytes in the interstitium
- Granuloma type: typical epithelioid-cell-granuloma formation with giant cells and lymphocytes
- Combined type: some atrophic epithelioid-cell-granulomatous and fibrous change
- Fibrotic type: the myocardial tissue replaced by fibro-hyaline changes, with sparse lymphocytic infiltration.
3. Phases of the lesion in cardiac sarcoidosis [36]
- Early (primarily lymphocytic) phase: areas indistinguishable from lymphocytic myocarditis
- Intermediate (primarily granulomatous) phase: active granulomatous lesion
- Late (primarily scar) phase: areas composed predominantly of scar

When we diagnose the resected heart for cardiac transplantation or autopsy, we should evaluate the patient as a whole. We should include any previous pathologic processes in our final diagnosis. We would interpret hearts without definitive granuloma as a case of cardiac sarcoidosis if there is presence of micro-granulomas and macroscopic distribution of the lesion. The macroscopic distribution of cardiac lesions in our cases matches very well with the radiologic findings. There were scattered macroscopic fibrotic scars in the ventricular septum, free wall, sub-epicardium, and papillary muscles without any matching distribution in the coronary arterial supply [10,18,26]. This random pattern was also present in the autopsied heart with classical histologic features of sarcoidosis. If radiologic diagnosis of sarcoidosis is based on the macroscopic distribution of the lesion, the same feature of the resected heart can form the pathologic basis of cardiac sarcoidosis when we diagnose cardiac sarcoidosis for whole heart specimen. The macroscopic distribution of the granulomas was described as patchy and involving the ventricular septum and the subepicardium [36,37]. These features are confirmed by radiologic studies [38–40].

Differential diagnosis of cardiac sarcoidosis from the pathologists' view was reviewed [1,24]. When we observe granulomas, we suspect sarcoidosis, giant cell myocarditis, and other granulomatous myocardial lesions, including those of infectious origin [20,21]. It may be necessary to broadly categorize these cases so that they are all classified into "granulomatous myocarditis," if any specific causes are detected. In our experience with cases involving clinically suspected cardiac sarcoidosis, the pathologists are asked if the case is compatible with cardiac sarcoidosis, and to carefully examine the pathologic specimens to find any suggestive features and any small micro-granulomas. This attitude will support cardiologists in considering cardiac sarcoidosis to ensure they do not miss the chance to start optimal treatment. There are several different patterns of myocardial inflammatory lesions [1,41]. In general, viral myocarditis involves the heart as a diffuse lesion. The lesion may involve some parts more severely, but the margin of the inflammatory lesion is indistinct. Rheumatic myocarditis also has distinct features of involvement of the valve, pericardium, and perivascular interstitium. Infectious myocarditis involves only the focus of involvement. Tuberculous lesions involve predominantly pericardium first, and the myocardial lesions are an extension of pericarditis. For sarcoidosis, nodular and mass-like involvement is characteristic and the intervening myocardium between granulomata is generally spared. It has to be emphasized however that the end-stage fibrotic lesion of cardiac sarcoidosis will show different shape. We value the endo-

myocardial biopsy to exclude other differential diagnoses from sarcoidosis in the absence of granulomatous inflammation.

Explant hearts for transplantation have similar concerns. When cardiac sarcoidosis is clinically diagnosed, pathologists will check for micro-granuloma. It is worthwhile to remind pathologists that granulomas may be indistinct in cardiac sarcoidosis after treatment. High suspicion for cardiac sarcoidosis is necessary in patients with end-stage heart failure with pacemaker or implantable cardiac defibrillator [42]. Cardiologists and surgeons may incorrectly diagnose dilated cardiomyopathy or ischemic cardiomyopathy when the case was not associated with rhythm disturbance. However, involvement of the conduction system is not the rule in cardiac sarcoidosis [1,18,24,25]. The pathologists' suspicion for cardiac sarcoidosis will require a search to find a granuloma or a micro-granuloma to confirm the diagnosis. It is also important for pathologists to examine the macroscopic morphology of the explant heart to find large confluent scars and fatty changes in the myocardium in both subendocardium and sub-epicardium.

Supplementary Information

The Data Supplement is available with this article at <https://doi.org/10.4132/jptm.2020.06.10>.

Ethics Statement

The study plan of this research was reviewed by the Seoul National University College of Medicine/Seoul National University Hospital (IRB NO. H-1802-050-921 and RESEARCH TITLE: Indirect pathological indicators for cardiac sarcoidosis on endomyocardial biopsy or the explant heart for transplantation). Review comments was: Since the risk of research is minimal, it is for expedited review, and the statement of reason for waiver of informed consent is reasonable. According to IRB Approval Criteria, the IRB approves the research. Further details of the rationale for this waiver was that this research was based on retrospective review of medical record and pathology slides, and anonymized case analysis. This analysis did not alter management of patients. See Attachment D: Informed consent and waiver of consent (<https://www.hhs.gov/ohrp/sac/rp-committee/recommendations/2013-january-10-letter-attachment-d/index.html>).

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Conflicts of Interest

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

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Prevalence of high-risk human papillomavirus and its genotype distribution in head and neck squamous cell carcinomas

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Background: High-risk (HR) human papillomavirus (HPV) is found in a subset of head and neck (HN) squamous cell carcinomas (SCCs). For oropharyngeal SCCs, HR HPV positivity is known to be associated with good prognosis, and a separate staging system for HPV-associated carcinomas using p16 immunohistochemistry (IHC) as a surrogate test has been adopted in the 8th American Joint Committee on Cancer staging system. We examined the HR HPV status and the genotype distribution in five HN subsites. **Methods:** Formalin-fixed paraffin-embedded tissue sections were used for p16 IHC and DNA extraction. HPV DNA detection and genotyping were done employing either a DNA chip-based or real-time polymerase chain reaction-based method. **Results:** During 2011–2019, a total of 466 SCCs were tested for HPV DNA with 34.1% positivity for HR HPV. Among HN subsites, the oropharynx showed the highest HR HPV prevalence (149/205, 75.1%), followed by the sinonasal tract (3/14, 21.4%), larynx (5/43, 11.6%), hypopharynx (1/38, 2.6%), and oral cavity (1/166, 0.6%). The most common HPV genotype was HPV16 (84.3%) followed by HPV35 (6.9%) and HPV33 (4.4%). Compared with HR HPV status, the sensitivity and specificity of p16 IHC were 98.6% and 94.3% for the oropharynx, and 99.2% and 93.8% for the tonsil, respectively. **Conclusions:** Using a Korean dataset, we confirmed that HR HPV is most frequently detected in oropharyngeal SCCs. p16 positivity showed a good concordance with HR HPV DNA for oropharyngeal and especially tonsillar carcinomas. The use of p16 IHC may further be extended to predict HR HPV positivity in sinonasal tract SCCs.

Key Words: Human papillomavirus; Head and neck; Squamous cell carcinoma; Oropharynx

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Human papillomavirus (HPV)-associated squamous neoplasia, from simple warts to squamous cell carcinoma (SCC), occurs in various skin and mucosal sites including the uterine cervix, anogenital area, and upper aerodigestive tract of head and neck (HN). Among HPV genotypes, those that can transform the infected cells to malignancy are designated 'high-risk' (HR) types, the most well-known of which is HPV16. For the uterine cervix, about 90% of SCCs are associated with HR HPV [1]. In the HN, HR HPV prevalence of SCCs varies among the subsites, the oropharyngeal tumors being predominantly associated with the virus [2,3].

HR HPV infection leads to overexpression of p16 protein as a consequence of viral E7-mediated degradation of retinoblastoma (Rb) protein [4]. Therefore, p16 immunohistochemistry (IHC)

in paraffin-embedded tissue has been utilized as a surrogate test for HPV-specific tests, such as detection of HPV DNA and RNA [5]. In the oropharynx, the prognosis of HR HPV-positive SCCs is known to be superior compared with that of HPV-negative tumors [6,7]. p16 positivity alone was also shown to be an independent prognostic factor of oropharyngeal SCCs [5,6], and the recent 8th American Joint Committee on Cancer (AJCC) staging system adopted a separate TNM staging system for 'HPV-mediated (p16 positive)' oropharyngeal carcinomas [8].

The incidence of oropharyngeal cancer, most of which are SCCs, has been reported to be increasing worldwide, and this is attributed to a rise in HPV-positive portion of oropharyngeal carcinomas in some studies [3,9-12]. As for the Republic of Korea, a National Health Insurance Service data-based study reported an

increase in the incidence of tonsillar cancer during 2002–2015 from 1.1 to 2.4 for men, and from 0.31 to 0.46 for women (per 100,000) [13]. On the other hand, data on HPV-positive fraction of Korean HN SCCs has been published for small cohorts of oropharynx and oral cavity tumors, with a wide range of values [14–24]. Using a recent (2011–2019) and large ($n = 466$) HN SCC dataset and employing HPV DNA polymerase chain reaction (PCR), we aimed to assess the HPV-attributable fraction in each subsite of the oropharynx, oral cavity, larynx, hypopharynx, and sinonasal tract.

MATERIALS AND METHODS

Case selection

The HN SCC dataset of this study were derived from two of Catholic Medical Center hospitals, Seoul St. Mary's Hospital and Bucheon St. Mary's Hospital. The pathology file of Seoul St. Mary's Hospital was searched for HN SCCs that underwent p16 IHC and/or HPV DNA PCR test as ancillary tests during the period from January 2011 to December 2019. A total of 717 HN SCC cases from 717 patients were retrieved, which includes small biopsies as well as resection cases. Bucheon St. Mary's Hospital dataset consists of 70 HN SCC cases accessioned from January 2011 to December 2019. Thirty-one of Bucheon St. Mary's Hospital cases had undergone p16 IHC and/or HPV DNA PCR at the time of diagnosis. For the remaining 39 cases for which p16 IHC and/or HPV PCR was not already done, paraffin-embedded tissue blocks were used to perform p16 IHC and HPV PCR. The paraffin blocks of Bucheon St. Mary's Hospital cases were loaned from Bucheon St. Mary's Biobank of The Catholic University of Korea (B2019091702). In total, 787 HN SCC cases from the two hospitals were analyzed. Included in the dataset were 28 cases for which p16 IHC and/or HPV PCR were not performed on the primary tumor but on the metastatic tumors of neck lymph nodes ($n = 23$) or on the recurrent tumors ($n = 5$). The p16 and HPV status of the primary tumor were assumed to be the same as in the metastatic/recurrent tumor [25] for these cases. Squamous cell carcinomas of unknown origin, nasopharyngeal carcinomas, and adenosquamous carcinomas were excluded.

p16 IHC

Formalin-fixed paraffin-embedded (FFPE) tissue sections of 4- μ m thickness were used for IHC employing Ventana Benchmark autostainer (Ventana Medical Systems, Tucson, AZ, USA) in each of the two hospitals. The p16 antibody used during the year 2011–2015 at Seoul St. Mary's Hospital was a mouse mono-

clonal antibody (JC8, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cases from year 2016 onward at Seoul St. Mary's Hospital and all of Bucheon St. Mary's Hospital were stained with a ready-to-use antibody of E6H4 clone (CINtec Histology, Ventana Medical Systems). p16 IHC result was considered positive when there was diffuse moderate or strong nuclear and cytoplasmic staining in more than 70% of the tumor cells.

PCR-based HPV DNA detection and genotyping

DNA was extracted from four 10- μ m-thick FFPE tissue sections of each case using QIASymphony DNA mini kit (Qiagen, Hilden, Germany) before 2016, and Maxwell 16 automated system (Promega Corp., Madison, WI, USA) later. HPV DNA detection and genotyping were done using either of the two commercial PCR-based kits. For cases accessioned before March 2018, a DNA chip-based genotyping kit (BMT HPV 9G DNA kit, Biometrix Technology, Chuncheon, Korea) was used. The later cases were tested using PANA RealTyper HPV Kit (PANAGENE Inc., Daejeon, Korea) that employs peptide nucleic acid probe-based multiplex real-time PCR and melting curve analysis. These tests were run at the department of pathology laboratories of Seoul St. Mary's Hospital, where Bucheon St. Mary's Hospital specimens were sent out. The HPV genotypes identified by the BMT HPV 9G DNA kit are 14 HR types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and five low-risk (LR) types (6, 11, 34, 40, 42), and the rest of HPV DNA are reported as 'other' types. The PANA RealTyper HPV Kit specifies 20 HR types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 70, 73, 82) and two LR types (6, 11) while reporting 18 LR types (30, 32, 34, 40, 42, 43, 44, 54, 55, 61, 62, 67, 74, 81, 83, 84, 87, 90) as 'other' types. Statistical analyses were done using Excel 2013 (Microsoft Corp., Seattle, WA, USA) and SPSS statistics ver. 20.0 (IBM SPSS, Armonk, NY, USA) programs. A p -value $< .05$, double-sided, was considered statistically significant.

RESULTS

Prevalence of HR HPV and p16 positivity in HN SCCs according to subsites

The dataset of 787 HN SCCs (Table 1) consisted of 271 oral cavity (34.4%), 257 oropharynx (32.7%), 156 larynx (19.8%), 76 hypopharynx (9.7%), and 27 sinonasal tract (3.4%) carcinomas. p16 IHC was done for 771 of 787 cases. HPV DNA PCR was run for 466 cases, including 16 cases devoid of p16 IHC results. p16 positivity was 29.6% (228/771) in HN SCCs being the

Table 1. Frequency of p16-positive and HR HPV-positive cases in head and neck subsites

Subsite	No.	p16 IHC ^a	p16+	HPV DNA PCR ^b	HPV+ ^c	HR HPV+	HR genotype (n)
Oral cavity	271	264	20 (7.6)	166	5 (3.0)	1 (0.6)	-
Tongue	185	180	15 (8.3)	115	2 (1.7)	0	-
Mouth floor	20	20	2 (10.0)	14	2 (14.3)	1 (7.1)	16 & 31 & 39 (1)
Retromolar trigone	16	15	1 (6.7)	10	0	0	-
Gingiva	17	17	0	6	0	0	-
Buccal mucosa	25	24	2 (8.3)	16	1 (6.3)	0	-
Hard palate	7	7	0	4	0	0	-
Mucosal lip	1	1	0	1	0	0	-
Oropharynx	257	252	184 (73.0)	205	154 (75.1)	149 (72.7)	-
Tonsil	206	202	157 (77.7)	161	130 (80.7)	127 (78.9)	16 (105), 16 & 18 (1), 16 & 52 (1), 18 (1), 33 (6), 35 (10), 58 (3),
Tongue base	36	35	22 (62.9)	31	19 (61.3)	19 (61.3)	16 (16), 33 (1), 58 (1), 69 (1)
Soft palate and uvula	6	6	1 (16.7)	5	1 (20.0)	0	-
Other oropharynx	9	9	4 (44.4)	8	4 (50.0)	3 (37.5)	16 (2), 35 (1)
Sinonasal tract ^d	27	25	7 (28.0)	14	5 (35.7)	3 (21.4)	16 (2), 69 (1)
Larynx	156	154	11 (7.1)	43	8 (18.6)	5 (11.6)	16 (3), 16 & 51 & 68 (1), 16 & 18 (1)
Hypopharynx	76	75	6 (8.0)	38	2 (5.3)	1 (2.6)	16 (1)
Total	787	771	228 (29.6)	466	174 (37.3)	159 (34.1)	16 (134), 35 (11), 33 (7), 18 (3), 58 (4)

Values are presented as number (%) unless otherwise indicated.

HR, high-risk; HPV, human papillomavirus; IHC, immunohistochemistry; PCR, polymerase chain reaction.

^aNumber of cases tested for p16 IHC; ^bNumber of cases that underwent HPV DNA PCR; ^cIncludes low-risk and 'other' genotypes; ^dNasal cavity and paranasal sinus.

most prevalent in oropharyngeal carcinomas (184/252, 73.0%) and secondly in sinonasal tract carcinomas (7/25, 28.0%). HR HPV PCR-positivity was detected in 34.1% (159/466) of HN SCCs, 93.7% (149/159) of which was oropharyngeal carcinomas. The HR HPV PCR-positivity among oropharyngeal carcinomas was 72.7%, comparable to its p16-positive rate (73.0%). The prevalence of HR HPV in other subsites were 21.4%, 11.6%, 2.6% and 0.6%, for sinonasal tract, larynx, hypopharynx, and oral cavity, respectively. Among the HR HPV genotypes, HPV16 was overall the most common (84.3%, 134/159). HPV35 and 33 were the second and third most common ones, detected at 6.9% (n = 11) and 4.4% (n = 7), respectively. HPV58 and HPV18, each, was observed at 1.9% (n = 3). HPV18 co-occurred with HPV16 in two of the three cases. Multiple genotypes were detected in seven cases as a combination of HR types (2 of 16 & 18; 16, 51 & 68) or both HR and LR types (16, 52 & 81; 16 & 34; 16, 31, 39 & 6; 58 & 11). HR HPV prevalence in the oropharynx was further stratified according to its four subsites of tonsil (palatine), base of tongue, soft palate, and other oropharynx (posterior wall or unspecified). The tonsil and base of tongue showed high rates of HR HPV positivity (78.9% and 61.3%, respectively) compared with soft palate (0%, 0/5) and other oropharynx (37.5%, 3/8). There were 15 cases (3.2%, 15/466) where only a LR (n = 5) or 'other' type (n = 10) was detected. Cases positive for 'other' type were detected with the chip-based genotyping kit, and the HPV type could include either a minority of HR type (26, 53, 69, 70,

73, and 82) or an LR type according to the manufacturer.

Comparison of p16 IHC results with that of HPV DNA PCR tests

The correlation of p16 overexpression with transcriptionally-active HR HPV infection is well known for oropharyngeal SCCs [5]. We calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value of p16 IHC in predicting HR HPV DNA positivity in 450 HN SCCs (Table 2). Differences in these measures were notable among HN subsites. The oropharynx (n = 200) demonstrated high rates for the sensitivity, specificity, and PPV: 98.6 and 94.3 and 98.0%, respectively. When limited to the tonsil (n = 157), the sensitivity even reached 99.2%, with the specificity of 93.8%. By contrast, the sensitivity was 0% for oral cavity carcinomas (n = 159), the only HR HPV-harboring carcinoma being p16-negative. Among the nine p16-positive oral cavity carcinomas, one was positive for LR HPV, and the rest was negative for HPV. The numbers of hypopharyngeal and sinonasal tract carcinomas were not large enough in our dataset to consider their 100% sensitivity values significant.

Proportion of HR HPV-positive tonsillar SCCs compared across time periods and between different studies

The incidence of HR HPV-associated oropharyngeal SCCs has been shown to be rising over the past few decades in various

Table 2. Concordance between p16 IHC results and HR HPV status

	No.	p16+	HR HPV+	p16+ and HR HPV+	p16- and HR HPV+	p16+ and HR HPV-	Sensitivity ^a (%)	Specificity ^a (%)	PPV ^a (%)	NPV ^a (%)
Oral cavity	159	9	1	0	1	9	0	94.3	0	99.3
Oropharynx	200	148	147	145	2 ^b	3	98.6	94.3	98.0	96.2
Tonsil	157	126	125	124	1	2	99.2	93.8	98.4	96.8
BOT	30	19	19	18	1	1	94.7	90.9	94.7	90.9
Larynx	42	4	5	2	3	2	40.0	94.6	50.0	92.1
Hypopharynx	37	1	1	1	0	0	100	100	100	100
Sinonasal tract	12	3	3	3	0	0	100	100	100	100
Overall	450	165	157	151	6	14	96.2	95.2	91.5	97.9

IHC, immunohistochemistry; HR, high-risk; HPV, human papillomavirus; PPV, positive predictive value; NPV, negative predictive value; BOT, tongue base; PCR, polymerase chain reaction.

^aPCR-detected HR HPV DNA positivity is used as the true positive; ^bHPV16 was detected in both cases.

parts of the world [9]. Our group had previously published a study on tonsillar SCCs diagnosed during 1994–2010 at Seoul St. Mary's Hospital [26], in which the proportion of p16-positive cases was 62.0% (n = 31 of 50). As p16 IHC is a reliable surrogate test for HR HPV in the oropharynx, we compared the proportion of p16-positive tonsillar SCCs across the time periods 1994–2010 and 2011–2019. The p16-positive fraction for 2011–2019, 80.6% (137 of 170), was significantly different from that of 1994–2010, 62.0% (chi-square test, $p = .008$), demonstrating an increase in HR HPV-positive tonsillar SCCs cases over a decade of time diagnosed in a tertiary care hospital. However, from 2011 to 2019, a significant increasing trend in the proportion of HR HPV-positive carcinomas was not detected (linear-by-linear association test, $p = .097$) (Fig. 1). Of note, the proportion of HR HPV-positive tonsillar carcinomas for Bucheon St. Mary's Hospital cohort of current study was 70.8% (17 of 24), not significantly different from 81.5% (110 of 135) of Seoul St. Mary's Hospital (chi-square test, $p = .269$).

Searching the literature for HPV-associated HN SCCs of Korea identified 15 studies, which are summarized in Table 3. Most of these studies dealt with time periods earlier than that of our study, from 1990s to 2000s. Studies of tonsillar SCCs employing HPV DNA PCR reported HR HPV prevalence ranging from 23.4% to 73.5%. A roughly bimodal distribution of previously reported HPV prevalence in tonsillar SCCs could be assumed, one peak at ~30% (n = 4) and the other at ~70% (n = 3). Those rates at 64.1, 73.1, and 73.5% [17,23,27] were not significantly different from the 78.1% of the current study (chi-square test, $p > .05$ for each) although derived from cohorts much earlier than (dated 2007 or prior) the current study. On the other hand, rates at 20%–30% level in four studies [18,19,22,28] were far lower compared with the others at 70% level. Another four studies of tonsillar or oropharyngeal SCCs documented p16 IHC re-

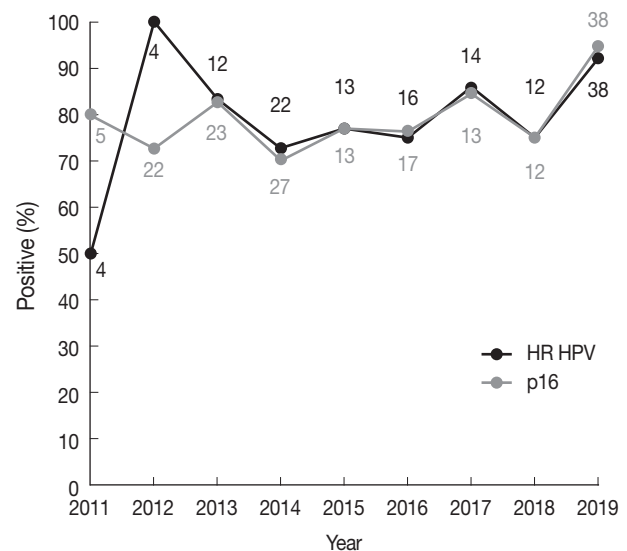


Fig. 1. High-risk (HR) human papillomavirus (HPV) prevalence and p16 positivity in tonsillar squamous cell carcinomas (SCCs) across years at diagnosis. Trends in the proportion of HR HPV-positive (black line) and p16-positive (grey line) newly diagnosed tonsillar SCCs for the Seoul St. Mary's Hospital cohort. Numbers above or below a datapoint indicate the total number of tests done in the corresponding year (black, HPV polymerase chain reaction; grey, p16 immunohistochemistry).

sults without performing HPV-specific tests; the p16-positive rates were all greater than 50%, from 62.0% to 82.5% [16,20,26,29]. For other HN subsites, reports of HR HPV prevalence were found for oral cavity and hypopharyngeal carcinomas (Table 3). The oral cavity HR HPV positivity (0.6%) of the current study was the least of all Korean studies while the sample size was the largest (n = 166).

DISCUSSION

The present study is the first to survey the HPV prevalence

Table 3. HR HPV prevalence of head and neck squamous cell carcinomas in Korean studies

Study	Site	Sampling period ^a	HPV detection method	HR HPV prevalence, n (%)	HPV genotype (%)
Oh et al. (2004) [27]	Tonsil	NS	PCR ^b	25/39 (64.1)	16 (92), 33 (4), 58 (4)
Kim et al. (2007) [17]	Tonsil	1995–2005	PCR	38/52 (73.1)	16 (89.5), 18 (2.6), 33 (2.6), 35 (2.6), 58 (2.6)
Kim et al. (2010) [18]	Tonsil	1999–2004	PCR	12/47 (25.5)	16 (100), 18 (8.3) ^d
Park et al. (2012) [23]	OP (tonsil, BOT, SP, P)	2002–2007	PCR, p16 ^c	OP (PCR): 53/93 (57.0) OP (p16): 46/93 (49.5) Tonsil (PCR): 36/49 (73.5)	16 (95.6), 18 (2.2), 33 (2.2)
Song et al. (2012) [26]	Tonsil	1994–2010	ISH, p16	ISH: 20/52 (38.5) p16: 31/50 (62.0)	NA
Lee et al. (2013) [20]	Tonsil	2000–2010	ISH, p16	ISH: 59/89 (66.3) p16: 74/89 (83.1)	NA
Kwon et al. (2014) [19]	Tonsil	1997–2010	PCR, p16	PCR: 28/79 (35.4) p16: 31/79 (39.2)	16 (100)
No et al. (2015) [22]	Tonsil	1998–2009	PCR	41/175 (23.4)	16 (43.9), 18 (43.9)
Kim et al. (2014) [28]	OP (tonsil, BOT, SP)	2004–2011	PCR, p16	OP (PCR): 21/74 (28.4) OP (p16): 35/74 (47.3) Tonsil (PCR): 15/47 (31.9) Tonsil (p16): 24/47 (51.1)	16 (76.2), 18 (28.6) ^d , 33 (4.7), 35 (4.7)
Lee et al. (2016) [29]	OP (tonsil, BOT, SP)	2001–2011	p16	104/126 (82.5) ^e	NA
Kim et al. (2016) [16]	OP (tonsil, BOT, SP, P)	2002–2013	p16	89/133 (66.9) ^e	NA
Current study	OP (tonsil, BOT, SP, P)	2011–2019	PCR, p16	OP (PCR): 149/205 (72.7) OP (p16): 184/252 (73.0) Tonsil (PCR): 127/161 (78.9) Tonsil (p16): 147/189 (77.8)	16 (83.9), 35 (7.4), 33 (4.7), 58 (2.7), 18 (1.3), 52 (0.7)
Joo et al. (2013) [40]	Hypopharynx	1996–2011	ISH	7/64 (10.9)	NA
Shin et al. (2002) [24]	Oral cavity	NS	HPV16, 18, 33-specific PCR	11/76 (14.5)	16 (36.4), 18 (72.7), 33 (18.2)
Lee et al. (2010) [21]	Oral cavity	1995–2005	PCR	12/36 (33.3)	16 (91.7),
Kim et al. (2010) [18]	Oral cavity	1999–2004	PCR	1/22 (4.5)	16 (100)
Joo et al. (2012) [14]	Oral cavity	1994–2009	ISH	6/90 (6.7)	NA
Kim et al. (2013) [15]	Oral cavity	2010–2012	PCR	2/86 (2.3)	16 (100)

HR, high-risk; HPV, human papillomavirus; NS, not specified; PCR, polymerase chain reaction; OP, oropharynx; BOT, base of tongue; SP, soft palate; P, other pharyngeal sites; ISH, HR HPV DNA in situ hybridization; NA, not applicable.

^aTime of diagnosis; ^bPCR for HPV DNA; ^cImmunohistochemistry; ^dIncluding co-occurrence with HPV16; ^ep16 positive rate as a surrogate for HPV-positive proportion.

and p16 positivity for SCCs of multiple HN subsites in Korea. HR HPV was predominantly found in oropharyngeal carcinomas, with 72.7% positivity. Meta-analysis studies have shown overall HPV prevalence of 40%–50% for oropharyngeal carcinomas globally, with the highest rates of 70%–75% belonging to North American and Northern European countries [10,30–32]. It is interesting that the oropharyngeal HPV positivity, 72.3%, of the current Korean dataset corresponds to the upper range of prevalence values observed in some Western populations and is higher than those of other Asian countries (mostly at about 50% or lower) [32,33]. Among oropharyngeal subsites in our series, HR HPV prevalence was highest in the tonsil (78.9%) and second highest in the tongue base (BOT) (59.4%) while zero in the soft palate/uvula. The prevailing association of HR HPV with lymphoepithelial subsites of oropharynx, the tonsil and to a lesser extent the BOT, is well established [33]. p16 positivity demonstrated a good concordance with HR HPV DNA in our series

of oropharyngeal carcinomas, especially for tonsillar carcinomas, reassuring the surrogate use of p16 IHC for classification of HPV-associated oropharyngeal carcinomas [8]. As p16 overexpression likely represents transcriptionally-active HPV infection, p16-negative HR HPV DNA positivity may suggest inactive infection or DNA contamination [34,35]. Such incidences were rare for oropharyngeal carcinomas in our series (1.6%, 2 out of 140 HR HPV-positive cases).

HR HPV has been recognized as a carcinogenic agent and also a favorable prognostic factor for oropharyngeal carcinomas, but the significance of HPV in non-oropharyngeal HN SCCs are controversial [7,36]. We observed the second highest HR HPV prevalence (21.4%) in sinonasal tract SCCs with a good concordance (100%) between p16 and HR HPV in a series of 12 cases. This corroborates the previous demonstration in sinonasal SCCs of 20%–30% HR HPV prevalence with reliable p16 IHC for HR HPV [37–39]. For oral cavity and laryngeal SCCs, the concor-

dance between p16 and HR HPV status was poor in the current study, which is also in line with previous findings [41]. We observed the lowest HR HPV positivity in oral cavity SCCs, at 0.6% out of 166 cases tested. This is lower than the rates reported in other Korean studies (Table 3) and the prevalence summarized for oral cavity carcinomas in meta-analysis studies (24%–58%) [42]. As we obtained a high rate of HR HPV positivity for oropharyngeal carcinomas backed by a good concordance with p16 positivity, the low rate in oral cavity carcinomas is not likely due to a low sensitivity of the HPV detection methods we used. One possibility for higher HR HPV rates of oral cavity carcinomas in other studies could be inadvertent classification of HR HPV-positive base of tongue cancers as oral cavity (mobile tongue) cancers. Additional larger cohort studies are awaited to evaluate the proportion of HR HPV-positive oral cavity SCCs in Korea.

We noted that the proportion of HPV-associated tonsillar SCCs significantly increased, from 62.0% to 79.5%, over a decade of time by comparing the p16-positive proportion of the previous and current case series from a single hospital. The seven earlier Korean studies documenting HPV DNA status in tonsillar SCCs showed either a rate comparable to the 78.9% of our series or a much lower rate at 20%–30% (Table 3). If not attributable to a lower sensitivity of the particular HPV detection method used in the latter group of studies, the explanation for the lower HPV prevalence may be sought in the cohort characteristics. HPV-positive oropharyngeal SCCs are known to be associated with certain epidemiologic factors, such as younger age at onset, higher socioeconomic status, less tobacco/alcohol consumption and a greater number of lifetime sexual partners in Western studies [43]. Two of the four studies reporting lower HPV prevalence provided p16 positivity rates that did not vary greatly from their HPV detection rates [19,28]; this supports the possibility of true heterogeneity in HPV prevalence among different Korean cohorts. Further studies using a large cohort with consideration of socioeconomic risk factors are warranted to examine the prevalence of HPV-associated tonsillar/oropharyngeal carcinomas in Korea.

HPV16 was the most common HR HPV genotype both among non-oropharyngeal HN SCCs (90%, 9/10) and among oropharyngeal SCCs (83.9%) (Table 1) in our dataset as it has been known [2,31]. For the oropharyngeal SCC series, the second most common HR HPV type was HPV35 (7.4%, 11/149). Literature on HPV types of oropharyngeal SCCs in other countries shows that HPV16 almost always comprised 85%–90% and that HPV33 and HPV35 frequently ranked second or third comprising usually less than 5% each [11,44–46]. In the six Korean tonsillar SCC studies reporting HPV genotypes (Table 3), HPV33 and/or

HPV18 were often second most frequent to HPV16. Of note is that the recently developed 9-valent HPV vaccine targets seven HR types including HPV16, 18, 31, 33, 45, 52, and 58, thus not likely offering protection against HPV35. More studies on HPV genotypes in Korean oropharyngeal carcinomas are anticipated to evaluate the prevalence of HPV35.

In conclusion, we have examined the prevalence of HR HPV in 466 HN SCCs according to the five subsites of oral cavity, oropharynx, larynx, hypopharynx and sinonasal tract. The oropharyngeal SCCs showed the highest rate of HR HPV positivity among the HN subsites at 72.3%, and the rate peaked at 78.9% when only tonsillar carcinomas were considered. The proportion of HR HPV-positive tonsillar carcinomas significantly increased over a decade of time in a single hospital. As it has been known, p16 immunopositivity showed a good concordance with HR HPV DNA for oropharyngeal and especially tonsillar carcinomas. The use of p16 IHC may further be extended to predict HR HPV positivity in sinonasal tract SCCs.

Ethics Statement

The study protocol was approved by the Institutional Review Board of the Catholic Medical Center (XC19SEDI0066), with a waiver of informed consent.

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Conflicts of Interest

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

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A retrospective cytohistological correlation of fine-needle aspiration cytology with classification by the Milan System for Reporting Salivary Gland Cytopathology

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Background: Before publication of the new classification system named the Milan System for Reporting Salivary Gland Cytopathology (MSRSGC) in 2018, there was no standard classification for salivary gland lesions obtained by fine-needle aspiration (FNA). We therefore aimed to evaluate the diagnostic utility of this system by retrospectively reviewing FNA samples using the MSRSGC and to determine their risk of developing into neoplasms and becoming malignant. **Methods:** Retrospective slide review and classification of salivary gland FNAs obtained over a 6-year period (2013–2018) at a single center were performed by two pathologists. The risks of neoplasm and malignancy for each category also were calculated. **Results:** This study surveyed 374 FNAs (371 patients) performed over a six-year period and selected 148 cases that included documented surgical follow-up (39.6%). Among the surgically treated cases, the distributions of FNA categories were as follows: non-diagnostic (ND; 16.9%), non-neoplastic (NN; 2.7%), atypia of undetermined significance (AUS; 3.4%), benign (BN; 54.7%), salivary gland neoplasm of uncertain malignant potential (SUMP; 10.1%), suspicious for malignancy (SM; 6.8%), and malignant (M; 5.4%). The risk of malignancy (ROM) was 24.0% for ND, 0% for NN, 40.0% for AUS, 2.5% for BN, 46.7% for SUMP, 100% for SM, and 87.5% for M. The overall diagnostic accuracy was 95.9% (142/148 cases). **Conclusions:** The newly proposed MSRSGC appears to be a reliable system for classification of salivary gland lesions according to the associated ROM.

Key Words: Salivary gland; Fine-needle aspiration; The Milan System for Reporting Salivary Gland Cytopathology; Risk of malignancy

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Salivary gland fine-needle aspiration (FNA) is a well-established, minimally invasive, and cost-effective procedure that rarely results in complications [1,2]. FNA provides crucial information for clinical management of tumors, such as by distinguishing between neoplastic and non-neoplastic lesions and benign and malignant lesions, as well as by providing samples for ancillary tests [3–7]. Clinical management and surgical interventions heavily depend on the information provided by FNA, along with clinical data and information obtained from imaging studies. It is crucial to differentiate between benign and malignant lesions. However, intratumoral heterogeneity and overlapping cytologic features of different salivary gland lesions hinder accurate subtyping of neoplasms [8–11]. Until recently, there has been no uniform reporting system for salivary gland lesions. A descriptive cytologic diagnosis without categorization can be confusing

for clinicians, who require more accurate information to establish an effective management plan [9].

In an attempt to address these challenges, an international group of pathologists and clinicians developed a tier-based classification system with the support of the American Society of Cytopathology and the International Academy of Cytology that was designated the Milan System for Reporting Salivary Gland Cytopathology (MSRSGC) [12,13]. The goal of this classification system was to standardize a reporting system for salivary gland lesions and to provide guidelines for their clinical management. Like other similar reporting systems, such as the Bethesda System for reporting thyroid cytopathology, this new classification system offers a clinically valuable framework for conceptualizing salivary gland lesions [14]. The MSRSGC is composed of seven categories: non-diagnostic (ND), non-neo-

plastic (NN), atypia of undetermined significance (AUS), benign neoplasm (BN), salivary gland neoplasm of uncertain malignant potential (SUMP), suspicious for malignancy (SM), and malignant (M).

Thus far, few studies have demonstrated promising use of this system. Therefore, in this study, we retrospectively applied the MSRSGC to categorize salivary gland FNA samples from 2013–2018. The objective of this study was to evaluate the diagnostic accuracy of the MSRSGC framework and evaluate this system's utility as a tool for risk assessment of salivary gland lesions.

MATERIALS AND METHODS

Research objective

A retrospective search of the cytopathology database from the past six years (January 2013–December 2018) for salivary gland (all major and minor salivary glands) FNA specimens at Gangnam Severance Hospital, School of Medicine, Yonsei University was performed. Clinical data regarding age, sex, and location of the lesion, as well as the type of tumor, were collected from patient medical records. Follow-up histopathological reports were also obtained if available. From January 2013 to December 2018, 374 FNAs were performed, and 150 of these also underwent surgical resection. Among them, 148 cases were finally enrolled in this study; two cases were excluded as they did not meet the inclusion criteria.

The FNAs were performed via a direct percutaneous or transoral route using a 23-gauge needle. The smears were then fixed in 95% ethanol for Papanicolaou staining, which was performed in the cytopathology laboratory.

Slide review and categorization

Blinded review of all FNA slides was carried out by two pathologists (J.H.P. and Y.J.C.), and each case was assigned to an MSRSGC category. When there was a diagnostic discrepancy, the two pathologists had a discussion to decide upon the best MSRSGC category. Category I cases were further divided into an inadequate group and a cyst-contents-only group. Matched slides of surgical specimens were also examined, and the histological diagnoses of these surgical specimens were categorized as NN, BN, or M.

Evaluation of risk of malignancy and risk of neoplasm

Cytologic-histologic correlations were performed to determine risk of malignancy (ROM) and risk of neoplasm (RON).

The ROM was defined as the ratio between the number of FNAs and the number of surgically confirmed malignancies. Similarly, the RON was defined as the ratio between the number of FNAs and the number of neoplasms, including both benign and malignant neoplasms. The ROM and RON values were calculated for each MSRSGC category.

RESULTS

Basal patient characteristics

This study included a total of 148 cases. The clinicopathologic characteristics of the patients are shown in Table 1. Our study population was made up of 64 (43.2%) male and 84 (56.8%) female patients, with a median age of 49 years (range, 11 to 85 years). FNA was performed predominantly from the parotid gland ($n = 120$, 81.1%). Thirty-four cases (23.0%) were confirmed as M neoplasms by histological categorization. Their categorization according to the MSRSGC is shown in Fig. 1.

Correlation between pathologic diagnosis and diagnosis based on MSRSGC categorization of FNA results

The preoperative cytological diagnoses and histological follow-up results are listed in Table 2. There were 25 cases with category I FNAs: eight NN lesions, 11 BNs, and six M neoplasms. There were four cases of category II FNAs: two NN lesions and two BNs. Among the five cases with category III FNAs, three involved pleomorphic adenomas and the other two were one

Table 1. Basal characteristics of patients

Characteristic	No. (%)
Sex	
Male	64 (43.2)
Female	84 (56.8)
Age (yr), mean $\pm$ SD (range)	50.0 $\pm$ 15.1 (11–85)
Location	
Parotid	120 (81.1)
Submandibular	28 (18.9)
MSRSGC category	
I	26 (16.9)
II	4 (2.7)
III	5 (3.4)
IV-A	81 (54.7)
IV-B	15 (10.1)
V	10 (6.8)
VI	8 (5.4)
Pathologic diagnosis category	
Non-tumor lesion	11 (7.4)
Benign neoplasm	103 (69.6)
Malignant neoplasm	34 (23.0)

MSRSGC, Milan System for Reporting Salivary Gland Cytopathology.

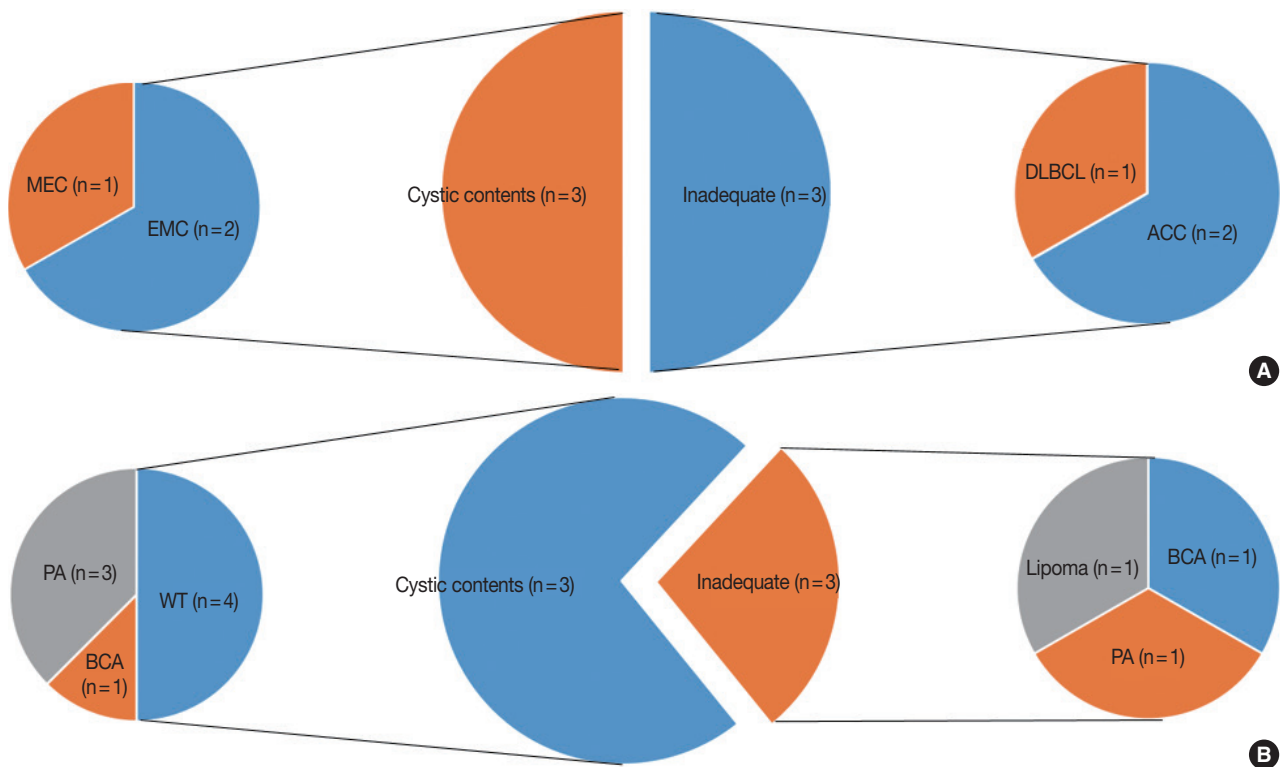


Fig. 1. Distribution of malignant (A) and benign (B) neoplasms in category I fine-needle aspiration samples. PA, pleomorphic adenoma; WT, Warthin tumor; MEC, mucoepidermoid carcinoma; EMC, epithelial myoepithelial carcinoma; ACC, acinic cell carcinoma; DLBCL, diffuse large B-cell lymphoma; BCA, basal cell adenoma.

case each of metastatic breast cancer in the intraglandular lymph node and diffuse large B-cell lymphoma. Notably, our results showed that 97.5% (79/81) of category IV-A cases were BN. However, the FNAs of one mucoepidermoid carcinoma and one epithelial myoepithelial carcinoma were placed into category IV-A. Among the category IV-B cases, seven were BN and seven were M neoplasms. In contrast, all category V cases involved malignant tumors, half of which were epithelial myoepithelial carcinomas. Among the eight category VI FNA cases, seven involved malignant neoplasms, and one was an atypical pleomorphic adenoma.

Further analysis of category I cases

Of all FNA cases that were followed by surgical resection, category I cases accounted for 17% (25/148). Of the 25 category I cases, 22 were located in the parotid gland, which is significantly higher than the remaining three cases that involved the submandibular gland. The lesions diagnosed as category I in the submandibular gland included fibrocalcific nodules, IgG4-related disease, and chronic sialadenitis. There were six category I FNA cases with lesions diagnosed as malignant tumors, which included two acinic cell carcinomas, two epithelial myoepithelial carci-

nomas, one diffuse large B-cell lymphoma, and one mucoepidermoid carcinoma. (Fig. 1). Moreover, there were 11 category I FNA cases with lesions diagnosed as benign tumors, including four pleomorphic adenomas, four Warthin tumors, two basal cell adenomas, and one lipoma. Only cystic contents without cells were aspirated in four cases involving Warthin tumors and three cases involving pleomorphic adenomas (Fig. 1B).

Risk stratification and comparisons with previous studies

We found that the RON was 68.0% for ND, 50.0% for NN, 100% for AUS, 100% for BN, 93.3% for SUMP, 100% for SM, and 100% for M. The corresponding ROM values were 24.0% for ND, 0% for NN, 40.0% for AUS, 2.5% for BN, 46.7% for SUMP, 100% for SM, and 87.5% for M. A summary of the ROM values obtained in the current study and those proposed by the MSRSGC and other studies is shown in Table 3.

Discrepant cases

A discrepancy between FNA and pathological diagnoses was observed in six cases (Table 4). In the NN FNA group (two cases), one case was diagnosed as a Warthin tumor and the other as a sialolipoma. Among the two cases classified as BNs using the

FNA samples, both were reported as malignant on resection (mucoepidermoid carcinoma and epithelial myoepithelial carcinoma). Moreover, among the cases classified as SUMPs, one was later diagnosed as a reactive lymph node (paracortical hyperplasia). Finally, among the cases categorized as malignant using

FNA samples, one was diagnosed as atypical pleomorphic adenoma upon histological follow-up (Fig. 2). The diagnostic accuracy achieved using FNA samples was 95.8% (115/120 cases) for the parotid gland and 96.4% (27/28 cases) for the submandibular gland. Thus, the overall diagnostic accuracy using FNA

Table 2. Categorization of pathological diagnoses of fine-needle aspirations based on the Milan System for Reporting Salivary Gland Cytology (MSRSGC)

MSRSGC category	Pathological diagnosis		
	Non-neoplastic (n = 11)	Benign neoplasm (n = 103)	Malignant neoplasm (n = 34)
I (n = 25)	Lymphoepithelial cyst (n = 4) IgG4-related disease (n = 2) Chronic sialadenitis (n = 1) Fibrocalcific nodule (n = 1)	WT (n = 4) PA (n = 4) Lipoma (n = 1) BCA (n = 2)	ACC (n = 2) EMC (n = 2) DLBCL (n = 1) MEC (n = 1)
II (n = 4)	Reactive lymph node (n = 1) Epidermal cyst (n = 1)	WT (n = 1) Sialolipoma (n = 1)	None
III (n = 5)	None	PA (n = 3)	Metastatic carcinoma (n = 1) DLBCL (n = 1)
IV-A (n = 81)	None	PA (n = 48) WT (n = 27) Oncocytoma (n = 1) BCA (n = 1) Myoepithelioma (n = 1) Atypical PA (n = 1)	MEC (n = 1) EMC (n = 1)
IV-B (n = 15)	Reactive lymph node (n = 1)	PA (n = 4) Oncocytoma (n = 1) Myoepithelioma (n = 1) Hemangioma (n = 1)	MEC (n = 3) EMC (n = 2) ACC (n = 1) Carcinoma ex PA (n = 1)
V (n = 10)	None	None	MEC (n = 5) AdCC (n = 2) ACC (n = 1) DLBCL (n = 1) Sqcc (n = 1)
VI (n = 8)	None	Atypical PA (n = 1)	MEC (n = 3) ACC (n = 2) Adenocarcinoma, NOS (n = 1) Metastatic melanoma (n = 1)

WT, Warthin tumor; ACC, acinic cell carcinoma; PA, pleomorphic adenoma; EMC, epithelial myoepithelial carcinoma; DLBCL, diffuse large B-cell lymphoma; BCA, basal cell adenoma; MEC, mucoepidermoid carcinoma; AdCC, adenoid cystic carcinoma; Sqcc, squamous cell carcinoma; NOS, not otherwise specified.

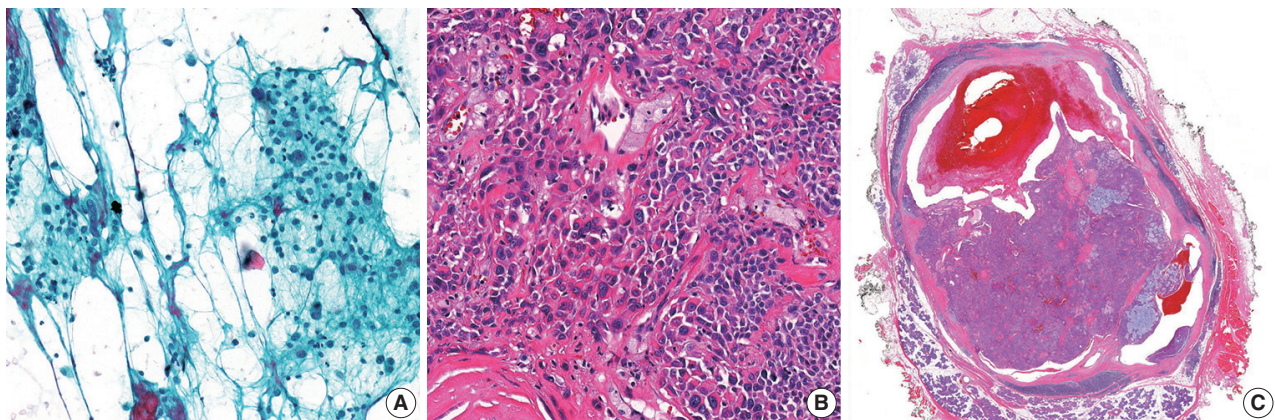


Fig. 2. Images of one of the false positive cases (atypical pleomorphic adenoma). (A) Fine-needle aspiration revealed highly atypical cells suspicious for malignant neoplasm. (B) Higher magnification of the mass showing atypical cells. (C) A lower magnification of the atypical pleomorphic adenoma without capsule invasion.

Table 3. Stratification based on risk of malignancy (%) in both current and previous studies

	ND	NN	AUS	BN	SUMP	SM	M
ROM proposed by the MSRSGC	25.0	10.0	20.0	<5	35.0	60.0	90.0
Current study	24.0	0	40.0	2.5	46.7	100	87.5
Rossi et al. [16]	17.0	16.0	53.0	6 ^a		79.0	100
Song et al. [14]	17.8	14.3	30.6	2.2	46.6	78.9	98.8
Viswanathan et al. [15]	6.7	7.1	38.9	5.0	34.2	92.9	92.3
Thirayai et al. [19]	8.5	1.6	0	1.9	26.7	100	100
Rohilla et al. [17]	0	17.4	100	7.3	50.0	-	96.0
Park et al. [18]	19.5	6.9	0	2.4	26.2	83.3	100

ND, non-diagnostic; NN, non-neoplastic; AUS, atypia of undetermined significance; BN, benign neoplasm; SUMP, salivary gland neoplasm of uncertain malignant potential; SM, suspicious for malignancy; M, malignant; ROM, risk of malignancy; MSRSGC, Milan System for Reporting Salivary Gland Cytopathology.

^aCases in the BN and SUMP categories were calculated together in this study.

Table 4. Cases showing a discrepancy between preoperative FNA diagnosis according to the MSRSGC system and the final pathologic diagnosis

No.	Type of discrepancy	MSRSGC category	Final pathologic diagnosis
1	False-negative	IV-A	Mucoepidermoid carcinoma, low-grade
2	False-negative	IV-A	Epithelial myoepithelial carcinoma
3	False-negative	II	Warthin tumor
4	False-negative	II	Sialolipoma
5	False positive	VI	Atypical pleomorphic adenoma
6	False positive	IV-B	Reactive lymph node

FNA, fine-needle aspiration; MSRSGC, Milan System for Reporting Salivary Gland Cytopathology.

samples was 95.9% (142/148 cases).

DISCUSSION

In this study, we reclassified FNA cases from a single center using the MSRSGC and correlated the results with those obtained using pathological diagnoses. Most salivary gland FNA cases that completed surgical follow-up were benign (103 cases; 69.6%), which is similar to the incidence reported by other studies [14-17]. The diagnosis rate of ND at our institution was 23.5%, with 68.0% RON and 24.0% ROM. Notably, the frequency of ND diagnosis was higher than the 10% frequency set by the MSRSGC.

The ROM of ND in our study was high compared to that of other studies [14-19]. Many factors are involved in an ND diagnosis, including aspiration technique, character of the lesion, method of specimen processing, and presence of artifacts from slide preparation [20]. Previous studies have suggested that rare, highly atypical cells could be placed in a category V if there is enough clinical suspicion, even if the number of cells to support this classification is insufficient [20].

In this study, we divided category I cases into subtypes and

examined their characteristics. Interestingly, 12.5% (4/32) of all Warthin tumors were placed in category I, and all four of these cases had only cystic contents. It is presumed that Warthin tumors could be accompanied by cystic degenerative changes in the center of the lesion. When category I was divided into two subtypes (inadequate and cystic contents only), there were no significant differences between the ROM values of the two subtypes (25% and 23.1%, respectively).

The ROM values for the NN and BN categories in our study were 0% and 2.5%, respectively, while the RON values were 50.0% and 100%. The ROM values for the NN and BN categories were lower than the proposed ROM incidence of 10% and lower than the 5% ROM proposed by the MSRSGC. In comparison, the ROM values for the NN and BN categories in other studies ranged from 1.6%–17.4% and from 1.9%–7.3%, respectively (Table 3) [14-19]. In our study, Warthin tumors and sialolipoma cases were reported in the NN category, which is in contrast to the findings of studies by Rossi et al. [16], Viswanathan et al. [15], and Song et al. [14], in which B-cell lymphomas predominantly accounted for false-negative diagnoses. The increase in ROM in the BN category in our study was attributed to one case of low-grade mucoepidermoid carcinoma and one case of epithelial myoepithelial carcinoma, which exhibited a similar distribution to that observed in the study by Rossi et al. [16]. Moreover, in the study by Song et al., the increase in ROM in the BN category was attributed to three cases of carcinoma ex pleomorphic adenoma and one case of adenoid cystic carcinoma [14].

According to the MSRSGC guidelines, cases in the AUS category should not exceed 10% of the total cases examined. In the current study, 3.8% of salivary gland FNAs were categorized as AUS, which is in accordance with this recommendation. The ROM for the AUS category was 40%, which is higher than the 20% proposed by the MSRSGC. However, one limitation of our study was that only five AUS FNAs were included,

precluding precise analysis of ROM for this type. In comparison, other studies have reported an ROM ranging from 0–100%. Moreover, various entities were included in this category, with pleomorphic adenoma being the most common.

The RON and ROM values for the SUMP category in our study were 93.3% and 46.7%, respectively, with pleomorphic adenoma and mucoepidermoid carcinoma being the most common benign and malignant diagnoses, respectively. The ROM was higher than the MSRSGC target rate and was similar to those reported in other studies (Song et al. [14] and Viswanathan et al. [15] reported ROMs of 46.6% and 34.2%, respectively). For the SM category, the RON and ROM values were 100% and 100%, respectively. Notably, the reported ROM for this category varies from institution to institution, ranging from 78.9%–100% [14–19]. This outcome is likely due to different institutional practices and disease populations, as well as differences in pathologist experience. Thus, this finding may represent a limitation of single-center studies. The RON and ROM values for the M category were 100% and 87.5%, respectively. The ROM in our study was slightly lower compared to that in the MSRSGC and other published studies [14–19]. In previous studies, squamous cell carcinoma was the most common malignant tumor in the M category [14–16]. However, the M category in our study consisted of only one case with squamous cell carcinoma; instead, mucoepidermoid carcinoma was the most frequently diagnosed malignancy. Compared to other studies, the limited sample size (eight cases) in our study may have led to both a lower ROM and greater differences in tumor type [14–19].

In this study, there were four false-negative and two false positive cases. Contributing factors might have included sampling errors, inadequacy of technique, vagueness in interpretation, and underestimation of low-grade malignant tumors. In particular, mucoepidermoid carcinomas with cystic changes are difficult to diagnose due to a high incidence of failure to gain optimal material [21,22]. A limited number of mucoepidermoid carcinoma cases contains all three cell types (mucous, intermediate, and squamous cells) [23]. However, among malignant tumors, mucoepidermoid carcinoma could often be relatively straightforwardly assigned to the SM category when mucus cells are present. One of the false positive cases in the present study involved an atypical pleomorphic adenoma; the FNA specimen showed several clusters composed of markedly atypical cells in the degenerated background (Fig. 2). In the resected specimens, except for focal cytologic atypia, there were no features that indicated malignancy. Rohilla et al. [17] reported a similar false-positive case. Given this intriguing case,

careful consideration of both the radiological findings and clinical assessments may help improve the predictive power of the MSRSGC.

In conclusion, we confirmed that the ROM at our institute was similar to the proposed ROM. Thus, the MSRSGC appeared to be effective in facilitating communication between pathologists and clinicians and may lead to a more comprehensive understanding of cytological diagnoses and establishment of appropriate treatment strategies.

Ethics Statement

This retrospective study was approved by the Institutional Review Board of Gangnam Severance Hospital (approval No. 3-2019-0219) with a waiver of informed consent due to its retrospective nature.

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Conflicts of Interest

SWH, a contributing editor of the *Journal of Pathology and Translational Medicine*, was not involved in the editorial evaluation or decision to publish this article. All remaining authors have declared no conflicts of interest.

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Renal intravascular large B cell lymphoma: the first case report in Korea and a review of the literature

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Herein, we describe the first case of renal intravascular large B cell lymphoma in Korea occurring in a 66-year-old female. She presented with mild fever and dyspnea. On physical and laboratory evaluations, hemophagocytic lymphohistiocytosis was suspected, but the bone marrow biopsy results were unremarkable. During the work-up, massive proteinuria developed, which led to a renal biopsy. The renal architecture was relatively well-preserved, but the glomeruli were hypercellular with the infiltration of atypical, large lymphoid cells with increased nucleus-cytoplasm ratio and clumped chromatin. Similar cells were also present in the peritubular capillaries. The tumor cells exhibited membranous staining for CD20 and CD79a. After the diagnosis of intravascular large B cell lymphoma, the patient received rituximab-based chemotherapy under close follow-up.

Key Words: Intravascular large B cell lymphoma; Kidney glomerulus; Peritubular capillary

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Intravascular large B cell lymphoma (IVLBL) is a rare, aggressive hematologic neoplasm characterized by the selective proliferation of neoplastic lymphoid cells within the lumina of small or intermediate sized vessels [1]. Most cases occur in adults, with a median age of 67 years [1]. Men and women are equally affected [2]. Clinically, IVLBL is often called ‘oncologist’s great imitator’, since it can affect various organs and manifest diverse symptoms according to the types of organs involved [3]. Central nervous system (CNS) and skin involvements are relatively frequent. However, renal involvement is rare [4]. In addition, fever, hepatosplenomegaly, pancytopenia, or even multiorgan failure may occur [5].

In this report, we described the first case of renal IVLBL in Korea occurring in a 66-year-old female. We also briefly reviewed 42 cases of renal IVLBL that were previously published.

CASE REPORT

A 66-year-old female was admitted to the Hematology Division of Internal Medicine with the presentation of a fever of 38°C, cough, non-bloody sputum, and dyspnea, which had started a

month previously. Her past medical history was unremarkable. Her blood pressure was 126/67 mm Hg, pulse rate was 79 beats per minute, and respiratory rate was 18 breaths per minute. The physical examination revealed no specific findings. Neither lymphadenopathy nor skin rashes were noted. The analysis of the complete blood cell count revealed anemia (hemoglobin, 7.1 g/dL) and thrombocytopenia (platelet count, $95 \times 10^3/\mu\text{L}$). Ferritin was 1,003.0 ng/mL. Serum haptoglobin level was normal and the Coombs test was negative. The C-reactive protein level was 86.7 mg/L and the lactate dehydrogenase level was 1,779 U/L. Blood urea nitrogen and serum creatinine levels were 2.8 mmol/L and 0.75 mg/dL, respectively. Serum aspartate transaminase was 31 IU/L and alanine transaminase was 10 IU/L. Total bilirubin level was mildly elevated (1.5 mg/dL). Antinuclear antibodies, anti-neutrophil cytoplasmic antibodies, anti-glomerular basement antibody, and hepatitis B and C viral markers were all negative. Sputum, blood, and urine cultures revealed all negative results. Chest computed tomography (CT) showed mild bronchiolitis in the right upper lobe field without definite evidence of pulmonary thromboembolism. Abdominal CT revealed mild hepatosplenomegaly. On the positron emis-

sion tomography–CT, diffuse ^{18}F -Fluorodeoxyglucose (FDG) uptake on the bilateral lungs, mild FDG uptake on the left lateral segment of the liver, and increased FDG uptake along the axial and appendicular bones were noted (Fig. 1). Clinically, hemophagocytic lymphohistiocytosis was suggested. A subsequent bone marrow biopsy revealed normocellular marrow without abnormal cell infiltration. Liver biopsy showed mild lobular hepatitis with Kupffer cell hypertrophy and increased hemophagocytosis.

During further work ups, heavy proteinuria (4 g/day and urine protein-to-creatinine ratio of 4.94) suddenly developed. A renal biopsy was performed on the twenty-fourth hospital day. The biopsy core of the kidney contained nineteen glomeruli. Most of the glomeruli were mildly enlarged and hypercellular with infiltration of atypical large lymphoid cells in the capillary lumina. They exhibited a high nucleus-cytoplasm ratio with size variation, chromatin clumping, and inconspicuous nucleoli. Immunohistochemical stains demonstrated strong CD20 and CD79a membranous positivity with a Ki-67 proliferation index of 80% (Fig. 2). The tubulointerstitium was relatively well-preserved, but the tumor cells were also present in some peritubular

capillaries. On electron microscopy, the glomerular capillary lumen was obliterated by swollen endothelial cells and infiltrating atypical lymphoid cells (Fig. 3). The diagnosis of renal intravascular large B cell lymphoma was confirmed. The patient is currently receiving chemotherapy consisting of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) with close follow-ups for three months.

DISCUSSION

IVLBL is a rare type of aggressive, non-Hodgkin B cell lymphoma that is confined to small to medium sized vessels. Clinically, two major patterns have been recognized [6]. The first pattern, called the ‘Classic form’, is prevalent in Western populations and usually demonstrates skin and CNS involvement and related symptoms. The second pattern, so called the ‘Asian variant’, is characterized by hemophagocytic syndrome, fever, multiorgan failure, hepatosplenomegaly, and bone marrow involvement. Our case exhibited clinical features of the ‘Asian variant’ with fever, hepatosplenomegaly, and nephrotic range proteinuria.

Renal involvement of IVLBL is infrequent. Forty-three cases, including the present case, have been reported in English literature (Table 1). There were 19 males and 21 females, ranging from 35 to 85 years of age (mean age, 60.5 years). The clinical signs of renal involvement were proteinuria (90.0%), some with nephrotic range (21.0%), renal failure (60.5%), and enlargement of the kidney upon radiologic evaluation (34.3%). Fifty percent of the cases also exhibited extrarenal involvement. Bone marrow involvement was identified in three out of 16 cases. Hemophagocytosis was observed in four cases, including the present case. All four cases occurred in Asians.

Histologically, malignant lymphoid cells demonstrate generally large, vesicular nuclei, one or more nucleoli, and scanty cytoplasm [1]. These cells usually express pan B cell markers including CD20, CD79a, and bcl-2, bcl-6, MUM-1, and variable expressions of CD5 and CD10 [7]. Although glomerular hypercellularity can be seen in hemophagocytic lymphohistiocytosis, cellular atypia and immunohistochemistry could differentiate malignant B lymphocytes from macrophages. Neoplastic cells lack surface molecules including CD29, CD54, and CD49d, facilitating the transvascular migration of the tumor cells. The aberrant expression of CXCR3 has also been described [8].

Of the 43 cases of intrarenal IVLBL with histologic descriptions, 35 cases exhibited the glomerular infiltration of tumor cells with or without peritubular and interstitial involvement. In four cases, the tumor cells were localized in peritubular cap-

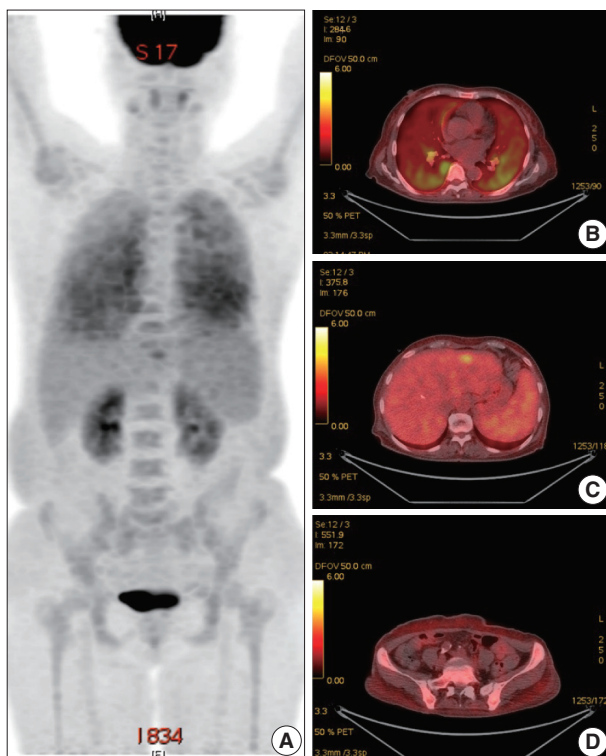


Fig. 1. Positron emission tomography (PET) images (^{18}F -Fluorodeoxyglucose PET). (A) Maximal intensity projection image. PET-computed tomography image of the lung (B), liver (C), and axial bones (D).

illaries without glomerular involvement. Our case showed glomerular and peritubular involvement. The microvascular infiltration and obliteration by tumor cells may be responsible for

proteinuria in our case through podocyte and endothelial injuries [9].

IVLBL generally shows aggressive clinical courses, often wors-

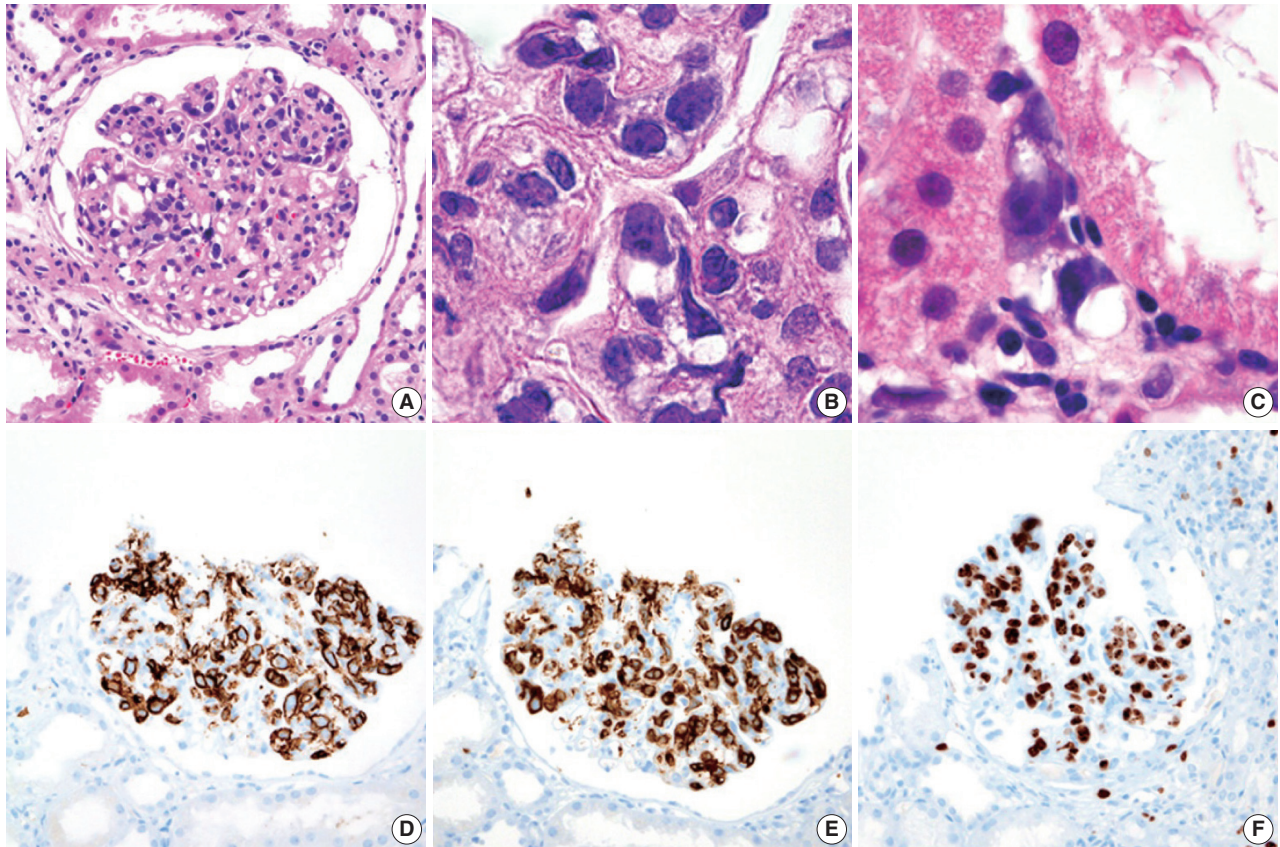


Fig. 2. Histopathologic findings and immunohistochemical staining results of renal intravascular large B cell lymphoma. (A) Atypical large lymphoid cells are confined to the lumina of the glomerular capillaries. (B) On higher magnification, malignant lymphoid cells exhibited nuclear size variation, chromatin clumping, and scant cytoplasm. (C) Some atypical lymphoid cells are scattered in the peritubular capillaries. On immunohistochemical staining, these atypical lymphoid cells demonstrated strong membranous positivity for CD20 (D) and CD79a (E). (F) The Ki-67 proliferation index was 80%.

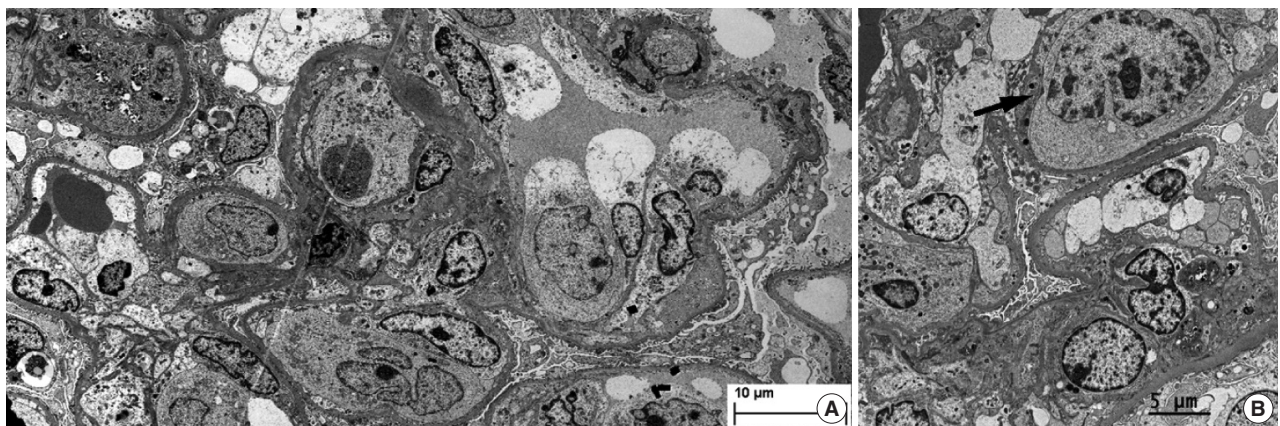


Fig. 3. Representative electron microscopy of the kidney. (A) Atypical large lymphoid cells are present in the glomerular capillary lumen, and the podocytes exhibit diffuse foot process effacement ($\times 1,500$). (B) On high power magnification, the atypical large lymphoid cell (arrow) shows prominent nucleoli and chromatin clumping ($\times 3,000$).

Table 1. Clinicopathologic characteristics of renal intravascular large B cell lymphoma

No.	Reference ^a	Sex	Age (yr)	Renal disease	Extrarenal involvement	Proteinuria	Fever	Bone marrow finding	Other disease	Radiologic evaluation	Lymphoma cell location	Outcome
1	Jothy et al.	N/A	N/A	Renal failure	N/A	+	N/A	N/A	N/A	N/A	G	N/A
2	Jothy et al.	N/A	N/A	Renal failure	N/A	+	N/A	N/A	N/A	N/A	G	N/A
3	D'Agati	F	62	Nephrotic syndrome	+	+	+	NR	-	Bilateral enlarged kidney	G/I	Died 1 mo after the biopsy
4	Avlesen et al.	F	60	Nephrotic syndrome	-	+	+	N/A	N/A	N/A	G	Alive (8 mo)
5	Nishikawa et al.	M	52	Renal failure	-	+	+	N/A	N/A	N/A	G	Alive (6 mo)
6	Agar et al.	F	70	Renal failure	-	+	N/A	N/A	N/A	N/A	G/P	N/A
7	Wood et al.	M	61	Renal failure	+	+	+	N/A	N/A	NR	G	Died 1 mo after the biopsy
8	Cheng et al.	F	35	N/A	+	N/A	+	Hemophagocytosis	Anemia	Bilateral enlarged kidney	G	N/A
9	Sepandj et al.	F	75	Renal failure	+	+	+	Erythroid hyperplasia and monocytosis	Anemia and monocytosis	Bilateral enlarged kidney	G/P	Alive (3 mo)
10	Charasse et al.	M	71	N/A	N/A	+	N/A	N/A	N/A	N/A	G	N/A
11	Kato et al.	F	64	Renal failure	+	+	+	N/A	N/A	NR	G	N/A
12	Kato et al.	M	65	Renal failure	+	+	+	N/A	N/A	Bilateral enlarged kidney	G/P/I	Post-mortem diagnosis
13	Kato et al.	M	82	Renal failure	+	-	+	N/A	N/A	NR	G	Post-mortem diagnosis
14	Shaknovich et al.	F	85	Renal failure	-	+	-	N/A	Depression and hypertension	NR	G/I	Alive (3 mo)
15	Jourdan et al.	F	49	N/A	-	+	N/A	N/A	N/A	N/A	G	N/A
16	Törnroth et al.	M	69	Renal failure	-	+	+	N/A	Urosepsis	Diffusely hyperechoic	G/P	Died 1 mo after the biopsy
17	Törnroth et al.	M	63	Renal failure	+	+	-	N/A	Deep vein thrombosis	NR	G	Died 21 months after the biopsy
18	Fozza et al.	F	53	Nephrotic syndrome	+	+	+	Hypercellularity and increased reticulin fiber	Pancytopenia	NR	G	Died 6 mo after the biopsy
19	Kakumitsu et al.	M	58	Nephrotic syndrome	+	+	+	Hemophagocytosis	HTLV-1 positive	NR	G/I	Alive (1.5 mo)
20	Ozolek et al.	M	72	NR	-	+	-	N/A	DCMP, AF	NR	G	Alive (3 mo)
21	Cossu et al.	F	56	Nephrotic syndrome	+	+	-	N/A	N/A	NR	G/I	Died 6 mo after the biopsy
22	Kusaba et al.	M	48	Renal failure	-	+	-	N/A	N/A	NR	G	Alive (24 mo)

(Continued to the next page)

Table 1. Continued

No.	Reference ^a	Sex	Age (yr)	Renal disease	Extrarenal involvement	Proteinuria	Fever	Bone marrow finding	Other disease	Radiologic evaluation	Lymphoma cell location	Outcome
23	Chroboczek et al.	M	56	NR	+	+	+	N/A	N/A	N/A	N/A	Alive (8 mo)
24	Dauchy et al.	M	67	Renal failure	+	+	+	N/A	N/A	NR	G	Died 9 mo after the biopsy
25	Sawa et al.	F	35	Renal failure	-	+	+	Hypercellular marrow	NR	Bilateral enlarged kidney	P	Alive (6 mo)
26	Balkema et al.	F	41	N/A	+	N/A	+	NR	NR	Bilateral enlarged kidney	N/A	N/A
27	Yoo et al.	F	74	Renal failure	-	+	+	NR	Anemia	NR	G/I	N/A
28	Niitsu et al.	M	52	Renal failure	-	-	-	NR	NR	Bilateral enlarged kidney	G/I	Alive (26 mo)
29	Kameoka et al.	F	40	N/A	-	+	-	N/A	N/A	NR	G	Alive (24 mo)
30	Yago et al.	M	76	Renal failure	+	+	+	N/A	N/A	Bilateral enlarged kidney	N/A	N/A
31	Deisch et al.	M	47	Renal failure	+	+	+	Rare atypical large cells	NR	NR	G/P	Alive (6 mo)
32	Bai et al.	M	41	Nephrotic syndrome	+	+	+	Immature cells of unknown origin	NR	Bilateral enlarged kidney	I/P	Alive (9 mo)
33	Kado et al.	F	72	Renal failure	-	N/A	+	N/A	N/A	Bilateral enlarged kidney	p	Alive (4 mo)
34	Iwagami et al.	F	78	Nephrotic syndrome	+	+	+	N/A	N/A	NR	G/P	Died 3 mo after the biopsy
35	Zhu et al.	M	55	NR	-	+	-	Hypercellular marrow	Anemia	NR	G	N/A
36	Kamalanathan et al.	F	77	Renal failure	-	+	-	N/A	HTN, sicca syndrome	NR	G	Alive (48 mo)
37	Hasegawa et al.	F	65	Renal failure	-	+	-	N/A	HTN	NR	G	Alive (109 mo)
38	Vankalakunti et al.	M	45	Renal failure	-	+	N/A	Hemophagocytosis	N/A	NR	P	N/A
39	Desclaux et al.	F	52	NR	-	-	+	NR	NR	Bilateral enlarged kidney	G/P/I	Alive (30 mo)
40	Kimura et al.	M	67	NR	+	-	+	Presence of malignant lymphoid cells	Anemia and thrombocytopenia	Bilateral enlarged kidney	P	N/A
41	Sekulic et al.	M	59	Nephrolithiasis	-	+	+	Hypercellular marrow without abnormal lymphoid cells	DM, HTN	NR	G/P	Died less than 1 mo after the biopsy
42	Pothen et al.	F	64	Nephrotic syndrome	+	+	+	N/A	HTN, CHO	NR	G	Alive (24 mo)
43	Current case	F	66	NR	-	+	+	Normocellular marrow without abnormal lymphoid cells	Hemophagocytosis	NR	G/P/I	Alive (3 mo)

N/A, not available; G, glomerulus; F, female; M, male; I, interstitium; NR, not remarkable; P, peritubular capillaries; HTLV-1, Human T-cell leukemia virus type 1; DCMP, dilated cardiomyopathy; AF, Atrial fibrillation; HTN, hypertension; DM, diabetes mellitus; CHO, hypercholesterolemia.

^aThe reference list is included in the Supplementary Material.

ened by the delay of diagnosis due to its nonspecific clinical presentation [10]. In terms of prognosis, at 6 months after diagnosis, 15 patients were alive and seven patients had died. Recently, rituximab-based chemotherapy has significantly improved clinical outcomes with a 3-year survival rate of 60%–80%. In addition, IVLBL only limited to the skin has been reported to exhibit better prognoses [11]. Our case is under rituximab-based chemotherapy for 3 months without further clinical deterioration.

In summary, we described a case of the renal involvement of IVLBL. Although the renal involvement of IVLBL is rare, this entity should be included as a differential diagnosis if unexplained hemophagocytic syndrome coupled with proteinuria persists.

Supplementary Information

The Data Supplement is available with this article at <https://doi.org/10.4132/jptm.2020.06.18>.

Ethics Statement

This work was approved by a faculty research grant of Yonsei University College of Medicine (4-2020-0150). The patient provided written informed consent for the publication of associated data and accompanying images.

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Conceptualization: HJJ. Data curation: MSK. Formal analysis: HJJ. Funding acquisition: HJJ. Investigation: MSK. Methodology: MSK, WIY, HRC. Project administration: HJJ. Resources: HJJ, WIY. Supervision: HJJ. Validation: HJJ, WIY, HRC. Visualization: HJJ, WIY, HRC. Writing—original draft: MSK. Writing—review & editing: HJJ, WIY, HRC. Approval of final manuscript: all authors.

Conflicts of Interest

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

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Breast implant–associated anaplastic large cell lymphoma: the first South Korean case

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To this date, breast implant–associated anaplastic large cell lymphoma (BIA-ALCL) has seldom been reported in the Asian population, despite the not insignificant number of breast implant surgeries performed locally. Here, we report the first case of BIA-ALCL in South Korea.

CASE REPORT

A 47-year-old woman presented to a local clinic who had previously undergone bilateral breast augmentation via transaxillary subpectoral augmentation (TSA) in 2013 for cosmetic reasons. The implanted device was a Natrelle silicone-filled textured breast implant (style 115, BIOCELL Textured Round Midrange Projection Gel-filled Breast Implant, 253 mL, Allergan, Dublin, Ireland). Six years postoperatively, in 2019, she noticed swelling of her right breast. An ultrasonographic examination revealed fluid collection and capsule discontinuity, suggesting a ruptured implant (Fig. 1A). On magnetic resonance imaging, however, implants were found intact, though fluid collection was prominent in the inner posterior side of the right breast (Fig. 1B).

Under the impression of implant rupture with seroma, aspiration was performed revealing large, atypical lymphocytes with irregular kidney-shaped nuclei, with occasional binucleation (Fig. 2A). These cells were confirmed as atypical T-cells positive for CD30 and CD3 and negative for anaplastic lymphoma kinase 1 per immunostaining, a typical phenotype of anaplastic lymphoma kinase (ALK)–negative BIA-ALCL (Fig. 2B, C) [1].

Whole-body positron-emission tomography (PET) showed

hypermetabolic capsule and fluid collection confined to the right breast. Blood test results were normal and bone marrow biopsy findings were negative for lymphoma involvement. With curative intent, the bilateral implants were removed with capsulectomy, which revealed irregular thickening of the capsule taken from the right breast with multiple focal areas of fibrinous deposits in the lining without distinct tumor masses (Fig. 3A). The implant did not show grossly identifiable foci of rupture. The capsule and implant from the left breast were unremarkable.

According to histologic sectioning, large atypical lymphocytes positive for CD30 but negative for ALK immunostaining that had infiltrated the luminal surface of the capsule from the right breast (Fig. 3B, C) were consistent with pathologic stage T2. Focal foreign body-type macrophages were identified throughout the specimen, denoting microscopic leakage (Fig. 3D). T-cell clonality was detected on gene rearrangement study for beta T-cell and gamma T-cell receptor genes. The contralateral breast was unremarkable, with a thin capsule and the absence of atypical lymphocytes.

DISCUSSION

BIA-ALCL is a rare type of T-cell lymphoma, occurring in recipients of either saline or silicone-filled textured type breast implants. BIA-ALCL is usually localized and the prognosis is excellent. Complete surgical removal of the capsule and implant with negative resection margins is curative in almost all patients. However, advanced-stage disease with metastasis has also been reported [2], which emphasizes the importance of the awareness of the entity and the making of an early diagnosis.

BIA-ALCL most commonly presents as effusion around the implant simulating rupture of the implant, while tumor mass formation is less frequent. The present case to our knowledge is

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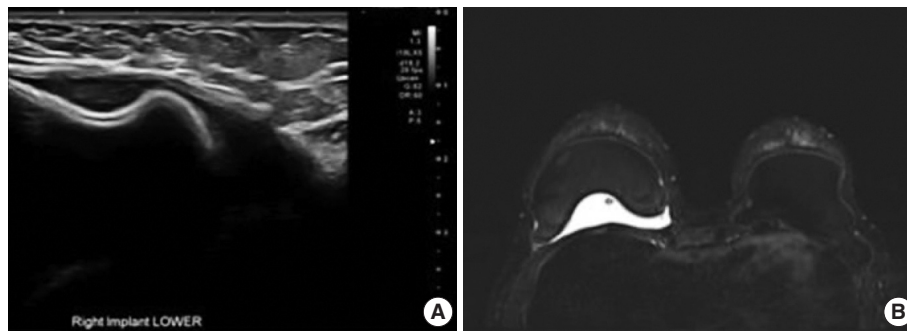


Fig. 1. Imaging studies. (A) Ultrasonography of the right breast shows suspected inner capsule discontinuity due to fluid collection, suspicious for rupture. (B) Magnetic resonance imaging of the breasts. Fat-suppressed T2-weighted axial image and dynamic contrast-enhanced T1-weighted axial image were processed. Fluid collection was identified at the inner side of the right breast capsule.

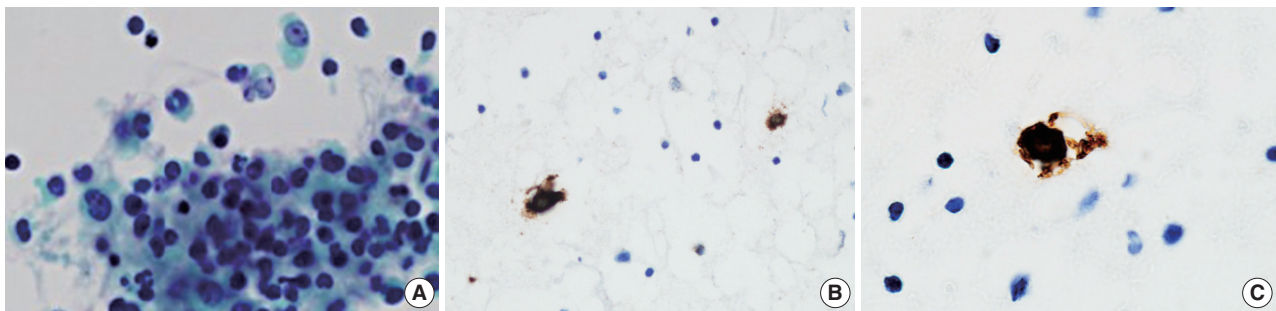


Fig. 2. (A) Large lymphocytes with binucleation and horseshoe-like nuclear indentation are visible. Tumor cells are admixed with mature, small lymphocytes for size comparison (SurePath Papanicolaou stain, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). (B) Large atypical cells positive for CD30 are admixed with mature lymphocytes. (C) Large neoplastic cells with membranous, Golgi staining pattern of CD30 immunolabeling.

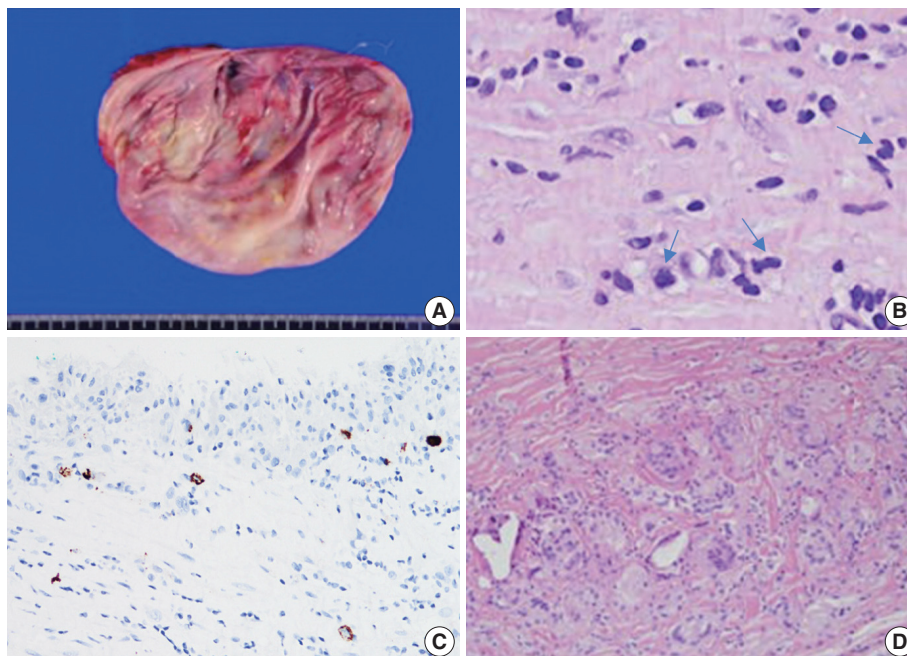


Fig. 3. The capsule removed from the right breast. (A) The capsule appeared irregularly thickened without definite “tumor” masses though with irregular, fibrin-like deposits in multiple foci. (B) Microscopically, atypical large lymphocytes with indented, kidney-shaped nuclei (arrows) had infiltrated the fibrinous material on the luminal side of the capsule. (C) The same cells show diffuse CD30 immunopositivity. (D) Foreign body-type giant cells were gathered around the deep surface of the capsule.

the first report of BIA-ALCL in a South Korean patient, with the typical presentation being similar to that in Western countries both clinically and pathologically. With 27,393 annual Allergan breast prostheses implanted in South Korea alone, one would expect a higher number of cases, so the apparent rarity of BIA-ALCL may be due to a trend of underdiagnosis. Due to the low index of suspicion, aspiration cytology is rarely performed in Korea for implant-associated effusions (personal observation, E.K.K.). The present case should raise a clinical awareness that BIA-ALCL does arise in Korean women and investigation to rule out BIA-ALCL should be performed in appropriate cases.

Ethics Statement

This case was deemed exempt by the Asan Medical Center Institutional Review Board (IRB #2020-0433). Informed consent was obtained from individual participant included in this study.

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Conceptualization: JRH, JL, CS. Data curation: EKK, HC, DHY, JRH, JL. Methodology: JRH, JL, GG. Writing—original draft: JRH, JL. Writing—review & editing: JRH, JL. Approval of final manuscript: all authors.

Conflicts of Interest

G.G., a contributing editor of the *Journal of Pathology and Translational Medicine*, was not involved in the editorial evaluation or decision to publish this article. All remaining authors have declared no conflicts of interest.

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Correction of author's name: Atypical femoral neck fracture after prolonged bisphosphonate therapy

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To the Editor,

We found an error in our published article.

Kim KK, Park YW, Kim TH, Seo KD. Atypical femoral neck fracture after prolonged bisphosphonate therapy. *Journal of Pathology and Translational Medicine* 2020; 54(4): 346-350. <https://doi.org/10.4132/jptm.2020.05.14>.

On page 346, the second author's name has been incorrectly spelled as Young-wook Park. The correct name is Yong Wook Park. We apologize for the error.

