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Pathologic Evaluation of Breast Cancer after Neoadjuvant Therapy

Cheol Keun Park · Woo-Hee Jung · Ja Seung Koo

Department of Pathology, Yonsei University College of Medicine, Seoul, Korea

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Corresponding Author
Ja Seung Koo, MD
Department of Pathology, Severance Hospital, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea
Tel: +82-2-2228-1772
Fax: +82-2-362-0860
E-mail: kjs1976@yuhs.ac

Application of neoadjuvant therapy (NAT) has become a more common breast cancer treatment due to the diversity and rapid development of associated therapeutic agents. NAT is currently established as a standard therapeutic approach for patients with large (> 2 cm) and locally advanced breast cancer. In addition, trials for NAT use in early-stage breast cancer are gradually increasing. Although there is no gain in survival benefit from NAT for breast cancer, it does offer several significant advantages over other modalities: (1) Response efficiency to a new therapeutic agent can be assessed because it is easy to detect a treatment response in a relatively short time period. In this respect, many clinical trials have been designed to evaluate NAT. Although there is no gain in survival benefit from NAT for breast cancer, it does offer several significant advantages over other modalities: (1) Response efficiency to a new therapeutic agent can be assessed because it is easy to detect a treatment response in a relatively short time period. In this respect, many clinical trials have been designed to evaluate NAT. (2) Patients with large cancers who show a response to NAT can undergo breast-conservation surgery. (3) The degree of response to NAT can play a role as a prognostic factor; one study reported that the rate of local recurrence depends on the extent of residual tumor after NAT. Given the potential benefits, exact assessment of breast specimens after NAT is very important. However, standard guidelines for pathologic evaluation of breast specimens after NAT have not been established. Herein, we offer a concise review of the various standard guidelines for pathologic assessment of breast cancer specimens after NAT.

Breast cancer, one of the most common cancers in women, has various treatment modalities. Neoadjuvant therapy (NAT) has been used in many clinical trials because it is easy to evaluate the treatment response to therapeutic agents in a short time period; consequently, NAT is currently a standard treatment modality for large-sized and locally advanced breast cancers, and its use in early-stage breast cancer is becoming more common. Thus, chances to encounter breast tissue from patients treated with NAT is increasing. However, systems for handling and evaluating such specimens have not been established. Several evaluation systems emphasize a multidisciplinary approach to increase the accuracy of breast cancer assessment. Thus, detailed and systematic evaluation of clinical, radiologic, and pathologic findings is important. In this review, we compare the major problems of each evaluation system and discuss important points for handling and evaluating NAT-treated breast specimens.

EVALUATION OF BREAST CANCER SPECIMENS AFTER NEOADJUVANT THERAPY

Specimen handling
Identification of the tumor bed is important for the handling of breast specimens after NAT. Close examination of fresh specimens cut into 5-mm sections or smaller is required for identification of the tumor bed. However, some cases require extensive sampling because of uncertainty in the gross identification of tumor bed. There have been attempts to insert metallic clips while conducting breast core biopsy for easy recognition of the tumor bed. However, this method cannot locate the tumor bed accurately because the inserted metallic clip shifts over time. Some guidelines suggest that small specimens (< 5 cm at the widest diameter or < 30 g) should be thinly sectioned and submitted in their entirety so that the specimens can be reintegrated upon histologic evaluation. However, these methods have limitations in that samples for research use cannot be secured. It is crucial to select representative sections when dealing with large specimens, such as those from a large lumpectomy or mastectomy. The important goal in specimen selection is to identify the area that correlates best with clinical and radiologic findings. This area, which is known as the pretreatment area, should com-
prise grossly identifiable tumor bed, a metallic clip, and peritumoral tissue. After slicing surgical specimens into ≤5-mm sections, the cross-section that includes the largest pretreatment area should be selected for sampling. The extent of tissue sampling varies according to guidelines: one or two tissue blocks from every 1 cm of pretreatment tumor or 10 blocks at least from an entire specimen. Because histologic patterns of residual post-NAT breast cancer tumors are diverse, different sampling methods can yield different evaluation results (Fig. 1), potentially resulting in sampling error. Even so, submission of large surgical samples in their entirety is not recommended because it is inefficient and offers little information despite the intensive sampling effort required. Thus, the extent of tissue sampling should be optimized and determined on a case-by-case basis considering clinical, macroscopic, and radiologic features. However, it is important, when creating sample specimens, to provide annotations and photographs of each tissue block to clarify the origin of tissue sections; this enables the pathologists conducting evaluations to identify correlations between macroscopic and histologic features. Also, exact descriptions, including the size of any grossly visible tumor beds and distances from resection margins, should be recorded.

Microscopic pathologic report

Pathologic variables that describe surgical breast cancer specimens that were not exposed to NAT are also important for post-NAT specimen. However, several factors should be taken into account, due to the diversity of evaluation systems for post-NAT breast cancer, including differences in major variables of each evaluation system and histologic factors causing post-NAT changes (Table 1). A summary of the pathologic reports for breast cancer after NAT is provided in Table 2.

Histologic tumor subtype and grade

In principle, the method to evaluate histologic subtype and tumor grade in breast cancer patients who received NAT is the same as that used for patients with non-neoadjuvant cancer. However, it is necessary to consider that NAT can affect histologic architecture, nuclear features, and tumor mitosis. Thus, some cases require comparison with pretreatment biopsy findings.

Tumor size and extent

There are many potential variables that can be used for assessing tumor size/extent in breast cancer patients who received NAT. Variable relevance depends on which tumor-response evaluation system is being used, because each system offers a different definition of significant tumor size. For example, the largest contiguous focus of the invasive cancer is the most important factor in determining ypT stage in ypTNM system. Contrarily, the two dimensions of the largest residual area of remaining invasive cancer are most important according to the Residual Cancer Burden (RCB) system. For the RCB system, however, the residual invasive cancer does not need to be contiguous, leading to a discrepancy in perceived tumor size between the two systems. The largest discrepancies in tumor size/extent due to the differences in measurement methods were observed when the tissue response pattern after NAT manifested as a scattered pattern (Fig. 2).

Tumor cellularity

Though NAT can affect several parameters of breast cancer, tumor cellularity is one of the most representative factors. Tumor cellularity is not always recorded in pathologic reports because it is important in some tumor-response systems but not in others. There are several factors that should always be considered when evaluating tumor cellularity. The first factor is the comparison of cellularity in pre- and post-treatment specimens (Fig. 3). Differences between pre- and post-treatment cellularity are important for some tumor-response systems; however, pretreatment cellularity is not considered in the RCB system. The second factor is tumor heterogeneity. Because residual tumor cellularity can appear heterogeneous after NAT, extensive tissue sampling should be performed. However, the majority of systems do not specifically include the cellularity of...
residual heterogeneous tumors, except for the RCB system, which recommends mentioning the average tumor cellularity.\textsuperscript{14}

**Lymphovascular invasion**

Lymphovascular invasion (LVI) is documented in most pathologic reports because it is a significant prognostic factor in non-neoadjuvant breast cancer.\textsuperscript{27,28} Though there are insufficient data on whether LVI is independently significant in neoadjuvant specimens, it should still be mentioned in pathologic reports.\textsuperscript{20} Ductal carcinoma in situ (DCIS) and LVI are defined as resistant breast cancer components after NAT.\textsuperscript{22} Therefore, in some situations, the only residual after NAT is tumor emboli in lymphovascular space, with no residual tumor in the breast parenchyma (Fig. 4).\textsuperscript{29} According to these guidelines, researchers have recommended that such cases not be regarded as pathologic complete response (pCR).\textsuperscript{20} Consequently, several LVI measurement methods have

| System | Included variable | Definition of pCR | Category status | Reference |
|--------|------------------|------------------|----------------|----------|
| AJCC (y) | Size of invasive carcinoma | No invasive carcinoma in breast and lymph node | Stage 0 | Boughey et al.\textsuperscript{8} |
| | Lymph node status (the number of metastatic lymph node and size of metastatic deposit) | | Stage 1 | |
| | | | Stage 2 | |
| | | | Stage 3 | |
| B-18 | Treatment effect in invasive carcinoma | No invasive carcinoma in breast and lymph node | No pathologic response | Diaz et al.\textsuperscript{26} |
| | Lymph node status (the number of metastatic lymph node and size of metastatic deposit) | | Pathologic partial response | |
| Miller-Payne | Presence of invasive carcinoma | No invasive carcinoma in breast | Grade 1: no change or some minor alteration in individual malignant cells, but no reduction in overall cellularity | Mamounas et al.\textsuperscript{9} |
| | Tumor cellularity | | Grade 2: a minor loss of tumor cells, but overall high cellularity; up to 30% reduction of cellularity | |
| | | | Grade 3: between an estimated 30% and 90% reduction in tumor cellularity | |
| | | | Grade 4: a marked disappearance of more than 90% of tumor cells such that only small clusters or widely dispersed individual cells remain | |
| | | | Grade 5: no invasive malignant cells identifiable in sections from the site of the tumor | |
| MNPI | Size of invasive carcinoma | No invasive carcinoma in breast and lymph node | MNPI=0.2×tumor size+lymph node stage+MSBR grade | Carey et al.\textsuperscript{10} |
| | Tumor grade | | Lymph node state: 1, node negative; 2, 1-3 positive; 3, ≥4 positive | |
| | | | Lymph node status (number of metastatic lymph node) | |
| Pinder | Tumor proportion (%) in remaining breast | No invasive carcinoma in breast and lymph node | Complete pathologic response | Ogston et al.\textsuperscript{11} |
| | Lymph node status (presence of evidence of response) | | Partial response to therapy | |
| | | | <10% of tumor remaining | |
| | | | 10%–50% of tumor remaining | |
| | | | >50% of tumor remaining | |
| | | | No evidence of response | |
| RCB | Size of tumor bed in two dimension | No invasive carcinoma in breast and lymph node | RCB 0: no residual disease | Abrial et al.\textsuperscript{12} |
| | Tumor cellularity | | RCB 1: minimal residual disease | |
| | Lymph node status (the number of metastatic lymph node and size of metastatic deposit) | | RCB 2: moderate residual disease | |
| | | | RCB 3: extensive residual disease | |

pCR, pathologic complete response; AJCC, American Joint Committee on Cancer; MNPI, Modified scores from Nottingham Prognostic Index; MSBR grade, Modified Scarff Bloom Richardson grade; RCB, residual cancer burden.
Surgical margins

Evaluation of resection margins is identical to that for non-neoadjuvant breast cancer specimens. Careful examination is required for evaluation of resection margins in neoadjuvant specimens because grossly invisible residual tumors or multiple scattered microscopic tumor foci are common. Furthermore, when the resection margin involves the tumor bed, it should be documented in the pathological report.

Table 2. Example of pathologic report form in breast cancer after neoadjuvant therapy

| Pathologic report form                                      |
|------------------------------------------------------------|
| Gross finding                                              |
| Residual identified tumor: yes/no                           |
| Quadrant of tumor                                          |
| Multifocality: yes/no                                       |
| Size of residual tumor: xx mm                              |
| Identified clip of marker: yes/no                           |
| Microscopic finding                                        |
| Histologic diagnosis: invasive carcinoma, NST               |
| Histologic grade: I/II/III (tubule score-nuclear grade-mitosis score) |
| Size of residual tumor bed: x mm                           |
| Size of the largest residual invasive carcinoma: x mm       |
| Residual tumor cellularity: %                              |
| Lymphovascular invasion: absent/present                     |
| DCIS component: yes/no                                      |
| Total tumor size including DCIS: x mm                      |
| Extensive intraductal component: yes/no                     |
| Type: cribriform/micropapillary/solid/papillary            |
| Nuclear grade: low/intermediate/high                        |
| Necrosis: absent/present (local/comedo)                    |
| ER/PR/HER-2 status: optional                               |
| Resection margin                                           |
| Invasive carcinoma: absent/present; distance to the closest margin |
| DCIS: absent/present; distance to the closest margin        |
| Tumor bed: absent/present                                   |
| Lymph node status                                          |
| Number of sentinel lymph nodes                             |
| Number of total axillary lymph nodes                       |
| Number of lymph nodes with macrometastasis                 |
| Size of largest metastasis: mm                             |
| Number of lymph nodes with micrometastasis                 |
| Number of lymph nodes with isolated tumor cells            |
| Number of lymph nodes with histologic evidence of treatment response but no tumor cells |
| Extracapsular extension: yes/no                             |
| Lymph node status                                          |
| Number of sentinel lymph nodes                             |
| Number of total axillary lymph nodes                       |
| Number of lymph nodes with macrometastasis                 |
| Size of largest metastasis: mm                             |
| Number of lymph nodes with micrometastasis                 |
| Number of lymph nodes with isolated tumor cells            |
| Number of lymph nodes with histologic evidence of treatment response but no tumor cells |
| Extracapsular extension: yes/no                             |

Evaluation of the axillary lymph node after NAT

The evaluation method for axillary lymph nodes is the same as that for non-neoadjuvant cases. Generally, all lymph nodes are sectioned into 2-mm intervals and sampled in their entirety for microscopic evaluation. Sometimes lymph nodes with complete treatment response are observed under microscopic evaluation for characteristic features, such as fibrous scarring, lymphocytic depletion, or histiocytic aggregation, without any identifiable tumor cell clusters (Fig. 5). If lymph nodes with these features are identified during microscopic evaluation, the total number observed should be noted in the pathologic report. When metastatic deposits are observed, the size of the largest metastatic tumor and presence/absence of extranodal extension should be recorded. It is difficult to measure the size of the largest metastatic tumor when the treatment response is accompanied by metastasis. In cases with multiple singly scattered tumor cells that involve the entire lymph node and when the treatment response is not accompanied by fibrosis, the size of the metastatic tumor is determined by measuring the size of the largest cell cluster. Some guidelines recommend measuring the sizes of the tumor cells and intervening stroma—not the largest cell cluster—when accompanied by a tumor response; consensus for these measurements has not been established among the various evaluation systems. Thus, when metastatic deposits are observed during microscopic evaluation, conditions such as macrometastasis, micrometastasis, and isolated tumor cells can be altered by changes in the sizes of metastatic deposits according to applied systems. However, residual disease in the lymph nodes is not considered pCR in most systems.
Pathologic complete response

Though each system that evaluates treatment responses to NAT has a unique definition of pCR, all systems record whether the patient has invasive carcinoma and whether it is identified in the breast parenchyma. 9-14 Significant differences among these evaluation systems are based on the inclusion or exclusion of DCIS and axillary lymph node status. Thus, description of DCIS and axillary lymph node status should always be included in pathologic reports because the treatment response evaluation systems differ across institutions.

Re-evaluation of biomarkers in breast cancer after NAT

Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2), which are representative biomarkers of breast cancer, should be used for evaluating invasive breast cancer; however, there is no consensus on whether ER, PR, and HER-2 status should be re-evaluated in breast cancer patients who received NAT. Different guidelines suggest different processes based on core biopsy results, because ER, PR, and HER-2 statuses after NAT are evaluated based on the biomarker status of pretreatment core biopsy. If ER, PR, and HER-2 statuses from pre-treatment core biopsy are all positive, there will be no changes in status for most patients; thus, re-evaluation is generally not recommended. However, re-evaluation is considered necessary in the following circumstances: (1) negative or equivocal results in core biopsy, (2) only DCIS or insufficient invasive carcinoma in core biopsy, (3) core biopsy performed at another institute, and (4) no treatment response. 20,21 Additionally, re-evaluation should be performed when the patient is enrolled in a clinical trial protocol or when ER, PR, or

Fig. 3. Comparison of tumor cellularity between pre-neoadjuvant therapy (NAT) and post-NAT. In comparison with the tumor cellularity of a pre-NAT biopsy (A), the tumor cellularity observed in a post-NAT surgical specimen (B) is significantly low.

Fig. 4. Residual tumor emboli in lymphovascular space after neoadjuvant therapy (NAT) (A, B). There are only tumor emboli in the lymphovascular space after NAT.
HER-2 status is unknown.

CONCLUSIONS

The number of existing post-NAT breast cancer specimens has recently increased because NAT is now established as an effective treatment approach for patients with large or locally advanced breast cancer and for cases of early-stage breast cancer. However, guidelines for pathologic evaluation of breast cancer after NAT have not been established; instead, there are several evaluation systems, each with different major main-effect variables. Moreover, from macroscopic examination to microscopic evaluation, there are several obstacles to pathologic evaluation of neoadjuvant breast cancer because there is a diverse range of histologic responses to NAT. Pathologic evaluation of residual disease is the most essential component of post-NAT breast cancer evaluation. Thus, the evaluation should be conducted based on close comparisons and correlations between clinical, radiologic, and pathologic findings.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Nuclear Expression of Hepatitis B Virus X Protein Is Associated with Recurrence of Early-Stage Hepatocellular Carcinomas: Role of Viral Protein in Tumor Recurrence

Jing Jin · Hae Yoen Jung · Kyu Ho Lee
Nam-Joon Yi · Kyung-Suk Suh
Ja-June Jang · Kyoung-Bun Lee

Departments of Pathology and Surgery, Seoul National University College of Medicine, Seoul, Korea

Background: Hepatitis B virus (HBV) plays well-known roles in tumorigenesis of hepatocellular carcinoma (HCC) in infected patients. However, HBV-associated protein status in tumor tissues and the relevance to tumor behavior has not been reported. Our study aimed to examine the expression of HBV-associated proteins in HCC and adjacent nontumorous tissue and their clinicopathologic implication in HCC patients. Methods: HBV surface antigen (HBsAg), HB core antigen (HBcAg), and HB virus X protein (HBx) were assessed in 328 HBV-associated HCCs and in 155 matched nontumorous tissues by immunohistochemistry staining. Results: The positive rates of HBsAg and cytoplasmic HBx staining in tumor tissue were lower than those in nontumorous tissue (7.3% vs. 57.4%, p < .001; 43.4% vs. 81.3%, p < .001). Conversely, nuclear HBx was detected more frequently in tumors than in nontumorous tissue (52.1% vs. 30.3%, p < .001). HCCs expressing HBsAg, HBcAg, or cytoplasmic HBx had smaller size; lower Edmondson-Steiner (ES) nuclear grade, pT stage, and serum alpha-fetoprotein, and less angioinvasion than HCCs not expressing HBV-associated proteins. Exceptionally, nuclear HBx-positive HCCs showed higher ES nuclear grade and more frequent large-vessel invasion than did nuclear HBx-negative HCCs. In survival analysis, only nuclear HBx-positive HCCs had shorter disease-free survival than nuclear HBx-negative HCCs in pT1 and ES nuclear grade 1–2 HCC subgroup (median, 126 months vs. 35 months; p = .015). Conclusions: Our data confirmed that expression of normal HBV-associated proteins generally decreases in tumor cells in comparison to nontumorous hepatocytes, with the exception of nuclear HBx, which suggests that nuclear HBx plays a role in recurrence of well-differentiated and early-stage HCCs.

Key Words: Hepatitis B X protein; Hepatitis B surface antigens; Hepatitis B core antigens; Carcinoma, hepatocellular

Hepatocellular carcinoma (HCC) is the third most fatal cancer worldwide and poses a major burden to the healthcare system. More than 50% of HCC cases overall and 70%–80% of HCC cases in hepatitis B virus (HBV)-endemic regions are attributable to chronic HBV infection. The mechanism of viral hepatitis-mediated induction of HCC involves the direct mutagenic effect of the virus on the host genome and the indirect effect of the inflammation-necrosis regeneration cycle in the setting of chronic hepatitis.

HBV is a member of the Hepadnaviridae family and has four important viral proteins: hepatitis B virus surface antigen (HBsAg), hepatitis B virus core antigen (HBcAg), hepatitis B virus X protein (HBx), and viral DNA polymerase. HBsAg and HBcAg are structural proteins that are the main components of the viral capsule and core. These proteins induce an immune response in infected hosts and can be used for the assessment of viral replication activity. Thus, they serve as clinical markers for diagnosis and follow-up of viral hepatitis patients via serum tests. HBx is a key regulatory nonstructural protein of the virus that is at the intersection of HBV infection, replication, pathogenesis, and carcinogenesis.

Integration of viral genomes in the host genome is considered a possible mechanism of hepatocarcinogenesis, and this notion is supported by the observation that a large portion of HCC have integrated HBV sequence encoding HBx and a truncated pre-S2/S protein. HBx protein regulates the cell cycle and DNA repair genes of host cells and induces cellular transformation via transactivation (protein interactions) in the nucleus and cytoplasm.
recurrence, and early antiviral treatment can increase disease-free survival (DFS) and overall survival of HCC patients. A specific mutated form of HBV known as genotype C was reported to be associated with HCC occurrence in cirrhotic patients. HBsAg positivity in non-neoplastic liver tissue was reported as a risk factor for HCC recurrence.

HBsAg expression is generally lower in tumor cells compared with adjacent non-neoplastic hepatocytes, and the HBx protein expression in non-neoplastic hepatic parenchyma is associated with the development of HCC in patients with chronic viral hepatitis. Previous studies of the expression of HBV genes and HBx protein in HCC and adjacent non-neoplastic hepatic parenchyma generally focused on the mechanisms underlying the HBV-related hepatocarcinogenesis.

The aim of this study was to assess the expression of the HBsAg, HBeAg, and HBx proteins in HCC and nontumorous liver tissue and to examine the histologic features of HCCs, possible correlations with hepatitis serum markers, and their influence on cancer prognosis.

**MATERIALS AND METHODS**

**Patients and clinicopathologic parameters**

We enrolled 328 HBV hepatitis patients who had been diagnosed with HCC based on a resected specimen and whose medical records and formalin-fixed paraffin blocks of tumor tissue were available from the archives of the Department of Pathology at Seoul National University Hospital (SNUH) from 1998 to 2004. We excluded co-infected hepatitis C virus hepatitis patients and patients who had neither serologic nor clinical evidence of HBV infection. The matched non-neoplastic hepatic parenchyma was available for 155 of the 328 patients. Clinical information, such as age, sex, surgical procedure, underlying etiology of liver disease, preoperative serum α-fetoprotein (AFP, μg/mL), preoperative treatment, and postoperative tumor recurrence, was collected from the medical records. Serological results of HBsAg, anti-HBs (HBsAb), IgG anti-HBc (HBcAb), hepatitis B virus e antigen (HBeAg), and anti-HBe (HBeAb) were based on the most recent preoperative tests from medical records. Depending on the state of the serum viral marker, liver function test, and clinical symptom of hepatic failure or portal hypertension (e.g., hypoalbuminemia, prolonged prothrombin time, ascites, hyperbilirubinemia, or hepatic encephalopathy), "asymptomatic carriers" had positive serum viral markers, but a normal liver function test and no clinical symptom of hepatic failure, while "noncirrhotic" patients had positive serum viral marker with abnormal liver function test, but no symptom of hepatic failure. Patients with symptom of hepatic failure were assigned to the cirrhotic patient group. Disease recurrence was defined as newly appearing lesions diagnosed by radiologic examinations such as ultrasonography and X-ray computed tomography, or based on serum tumor markers such as AFP, after an operation. Pathologic information, such as tumor size, number of tumors, gross type (vaguely nodular, expanding nodular, nodular with perinodal extension, and multinodular confluent), angioinvasion, large vessel invasion, Edmondson-Steiner (ES) nuclear grade, histologic pattern of the tumor, cellular type of tumor cells (hepatic vs. non-hepatic including giant, pleomorphic, spindle, and clear cell types), and extent of tumor invasion, was collected from pathology reports and review of the slides. Criteria for pathologic T stage (pT) followed the liver tumor staging of the American Joint Committee on Cancer, seventh edition. Clinico-pathologic parameters were assessed according to the general rules for examining primary liver cancer.

Of the 328 patients, 283 were male and 45 were female (M:F ratio of 6.3:1), with a median age of 55 years (range, 25 to 80 years). The mean size of a tumor was 5.44 cm (range, 0.8 to 24.0 cm). Most patients (92.1%, 302/328) had undergone partial hepectomy, such as right or left lobectomy, caudate lobectomy, or segmentectomy, whereas the remaining patients (7.9%, 26/328) had undergone total hepectomy for transplantation. Follow-up periods ranged from 0 to 161 months (median, 51 months). This study was approved by the Institutional Review Board of Seoul National University Hospital (H-1011-046-339).

**Tissue microarray construction**

Hematoxylin and eosin slides were reviewed, and one representative formalin-fixed paraffin-embedded (FFPE) archival block was selected for each case. Each core tissue biopsy (2 mm in diameter) was taken from individual FFPE blocks (donor blocks) and arranged in recipient paraffin blocks (tissue array blocks) using a trephine. Immunohistochemical studies were performed on 13 array blocks containing 328 HCC samples and on six array blocks containing 155 samples of HCC-adjacent non-neoplastic tissue as a healthy control (Superbiochips Laboratories, Seoul, Korea). Each tissue microarray had four cores of normal liver, normal bile duct, and normal gastrointestinal tract mucosa as a negative control.

**Immunohistochemistry and interpretation**

Sections (4 μm) were stained for HBeAg (1:800, hepatitis B core antigen rabbit polyclonal antibody, Cat. No. B0586, DAKO,
Copenhagen, Denmark), HBsAg (1:200, HBsAg mouse monoclonal antibody, clone number S1-210, Cell Marque, Rocklin, CA, USA), and HBx (1:100, HBx mouse monoclonal antibody, clone number 3F6-G10, Thermo, Rockford, IL, USA) after antigen retrieval using a microwave and pH 6.0 citrate buffer. The slides were stained according to methods specified in the UltraVision LP kit (Lab Vision, Fremont, CA, USA) or the Envision kit (Dako, Glostrup, Denmark) using the Bond polymer Refine Detection kit (Leica, Wetzlar, Germany). Unequivocal cytoplasmic staining for HBsAg in more than 5% of tumor cells was considered positive, and unequivocal nuclear and cytoplasmic staining for HbcAg in more than 5% of tumor cells was considered positive as described in a previous study. For HBx, both cytoplasmic and nuclear staining were observed in tumorous and nontumorous liver tissue; thus, we separately assessed the cytoplasmic and nuclear expression of HBx. The intensity of cytoplasmic staining was graded as weak, moderate, or strong by two pathologist (H.Y. Jung and K.-B. Lee), and a grade higher than moderate was considered as positive criteria. Nuclear staining was assessed by image analysis of digitally scanned slides (Nuclear V9 algorithm, ScanScope, Aperio Technologies, Vista, CA, USA). The intensity of nuclear staining was graded as 1+ (intensity range, 230 to 210), 2+ (intensity range, 210 to 188), or 3+ (intensity range, 188 to 162), and more than 5% of tumor cells with more than 2+ intensity were considered as positive criteria.

Statistical analysis
Comparative analysis of HBsAg, HbcAg, and HBx expression with clinicopathologic parameters was assessed using the chi-square test. Survival analysis was performed using the Kaplan-Meier method. The results were considered statistically significant when p-values were < .05. All calculations were performed using the PASW statistics ver. 18.0 (SPSS Inc., Chicago, IL, USA).

Table 1. The percentage of patients positive for HBV proteins in the hepatocellular carcinoma and non-tumorous liver

|       | Tumor (n=328) | Non-tumor (n=155) | p-value |
|-------|--------------|-------------------|---------|
| HBsAg | Negative     | 304 (92.7)        | 66 (42.6) | <.001* |
|       | Positive     | 24 (7.3)          | 89 (57.4) | .632   |
| HbcAg | Negative     | 292 (89.0)        | 141 (91.0) |         |
|       | Positive     | 36 (11.0)         | 14 (9.0)  |         |
| HBx,nu| Negative     | 157 (47.9)        | 108 (69.7) | <.001* |
|       | Positive     | 171 (52.1)        | 47 (30.3)  |         |
| HBx,cyto| Negative    | 186 (56.7)        | 29 (18.7)  | <.001* |
|       | Positive     | 142 (43.3)        | 126 (81.3) |         |

Values are presented as number (%).
HBV, hepatitis B virus; HBsAg, HBV surface antigen; HbcAg, HBV core antigen; HBX, HBV X protein; nu, nuclear staining; cyto, cytoplasmic staining.
*p<.05.
Although nuclear HBx was more frequently expressed in HCCs with high ES nuclear grade, the histologic pattern of HCCs with nuclear HBx expression was mostly trabecular and retained the hepatic cell type \( (p = .002 \text{ and } p = .086, \text{ respectively}) \) (Table 2). Age and sex were not different between HBV protein positive groups and negative groups (data not shown).

**Correlation between serum hepatitis B markers and expression of HBV proteins in HCC**

We set out to test whether the serum level of hepatitis B markers or the expression profile of nontumorous liver tissue showed any correlation with the expression of HBV proteins in HCCs. All patients with HBsAg-positive HCCs were HBsAg positive in serum. Patients with positive serum HBeAb results showed lower positive rates of HBsAg in HCCs than did patients with negative serum HBeAb \( (10.8\% \ [7/65] \text{ vs } 1.8\% \ [2/108], p = .027) \). Expression of HBcAg and expression of nuclear HBx in HCCs were not significantly concordant with the serum status of HBsAg, HBsAb, HBcAb, HBeAg, and HBeAb. As for the clinical stage of hepatitis, nuclear HBx was more frequently expressed in HCCs of noncirrhotic chronic hepatitis patients compared to asymptomatic carriers or cirrhotic patients \( (\text{noncirrhotic vs asymptomatic vs cirrhosis; } 75\% \ [15/20] \text{ vs } 35\% \ [13/37] \text{ vs } 43.9\% \ [54/123], p = .023) \).

**Progression of HBsAg-, HBcAg-, and HBx-expressing HCC**

To test whether the HBsAg, HBcAg, or HBx status of tumors influences cancer progression, we analyzed DFS time after operation in the 328 patients with HBV-associated HCC using Kaplan-Meier analysis. We could not determine any correlation between DFS time and the expression status of HBsAg, HBcAg, and HBx in tumors and nontumorous tissue or the serum levels of hepatitis markers (data not shown). The size and multiplicity of the tumor, angioinvasion, ES nuclear grade, and the extent of...
Table 2. Clinicopathologic characteristics of hepatocellular carcinomas expressing HBV proteins

| Variable                        | HBsAg | HBx
|--------------------------------|-------|-------|
|                                 | No.   | Negative | Positive | No.   | Negative | Positive |
| Size (cm)                       | n=328 | n=304    | n=24     | n=292 | n=36     | n=30     |
| ≤3                              | 48.5% | 99/204   | 16/24    | 43    | 33.3%    | 12/36    |
| >3                              | 51.5% | 12/22    | 12/24    | 47    | 66.7%    | 24/36    |
| p-value                         | .005  | .290     | .005     | .44   | .005     | .006     |
| Angioinvasion                   | .010  | .813     | .010     | .63   | .001     | .001     |
| Absent                          | 16/171| 96/171   | 1/2      | 31/13| 13/2     | 13/2     |
| Present                         | 16/212| 5/0      | 15/2     | 3/0   | 12/2     | 12/2     |
| p-value                         | .017* | .648     | .017*    | .042 | .017*    | .161     |
| Large vessel invasion           |       |          |          |       |          |          |
| Absent                          | 312/157| 288/157| 24/100   | 277| 94.9%    | 35/97.2  |
| Present                         | 16/171| 16/53    | 0/0      | 15/51| 15/1     | 13/7.8   |
| p-value                         | .618  | .109     | .005     | .017| .005     | .008     |
| ES nuclear grade                |       |          |          |       |          |          |
| 1                               | 30/117| 21/66    | 9 (37.5)| 22/7.5| 8/22.2   | 21/13.4  |
| 2                               | 170/142| 159/142| 11/45.8 | 151/51| 19/52.8 | 83/52.9  |
| 3                               | 120/185| 116/185| 4 (16.7)| 111/38| 9/25.0   | 47/29.3  |
| 4                               | 8/66  | 8/24     | 0/0      | 8/2.7 | 0/0      | 6/3.8    |
| p-value                         | .001  | .018     | .008*    | .008| .008     | .008     |
| Histologic pattern              |       |          |          |       |          |          |
| Trabecular                      | 294/157| 272/157| 22/91.7 | 260 | 89.0%    | 34/94.4  |
| Nontrabecular                   | 34/64  | 32/10.5  | 2/8.3   | 32/11.0| 2/5.6    | 25/15.9  |
| p-value                         | .734  | .316     | .002*    | .640| .002     | .002     |
| Cellular type                   |       |          |          |       |          |          |
| Hepatic                         | 312/157| 289/157| 23/91.7 | 280 | 95.9%    | 32/88.9  |
| Nonhepatic                      | 16/64  | 15/4.9   | 1/4.2   | 12/4.1| 1/1.1    | 11/7.3   |
| p-value                         | .867  | .085     | .886     | .130| .006     | .019     |
| pT (AJCC 7th ed)                |       |          |          |       |          |          |
| 1                               | 132/157| 117/157| 15/62.5 | 120| 41.1%    | 12/33.3  |
| 2                               | 137/157| 129/157| 8/33.3  | 117| 40.1%    | 20/55.6  |
| 3                               | 42/142| 41/142   | 1/4.2   | 39/13.4| 3/8.3    | 20/12.7  |
| 4                               | 17/66  | 17/66    | 0/0      | 16/5.5| 1/2.8    | 7/4.5    |
| p-value                         | .013* | .334     | .914     | .019| .019     | .019     |
| Preoperative treatment          |       |          |          |       |          |          |
| Not done                        | 203/157| 189/157| 14/48.3 | 175| 59.9%    | 28/77.8  |
| Done                            | 125/157| 115/157| 10/41.7 | 117| 40.1%    | 8/22.2   |
| p-value                         | .709  | .037*    | .343     | .036| .036     | .036     |
| Serum AFP (μg/mL)               |       |          |          |       |          |          |
| ≤20.0                           | 187/157| 170/157| 17/70.8 | 160| 54.8%    | 27/75.0  |
| >20.0                           | 141/157| 134/157| 7/29.2  | 132| 45.2%    | 9/25.0   |
| p-value                         | .113  | .015*    | .090     | .001| .001     | .001     |

Values are presented as number (%).
HBV, hepatitis B virus; HBsAg, HBV surface antigen; HBcAg, HBV core antigen; HBx, HBV X protein; nu, nuclear staining; cyto, cytoplasmic staining; ES, Edmondson-Steiner; AJCC, American Joint Committee on Cancer; AFP, a-fetoprotein.
*p<.05.

Tumor invasion were statistically significant prognostic factors for tumor recurrence in the entire sample of 328 patients (data not shown). The low frequency of positive HBsAg in HCCs and well-differentiated histology of HBsAg-expressing HCCs may be a confounder of the above-mentioned negative results in the entire group, so we stratified the study group and analyzed progression-free survival in 99 patients with early-stage (pT1) and well-differentiated HCC (ES nuclear grade 1 and 2); these patients were a relatively low-risk group for HCC recurrence. In 48.5% (48/99) of the patients, the tumor recurred and median DFS time was 84 months, ranging from 2 to 153 months. As shown in Table 3, nuclear HBx in tumors was a statistically sig-
significant prognostic factor (p = .015), whereas the presence of HBsAg in a tumor showed a tendency for poor prognosis of early-stage HCCs, but did not reach statistical significance (p = .095). Cytoplasmic expression of HBx in tumor and nontumorous tissue was not associated with tumor recurrence. In nontumorous liver tissue, HBsAg-, HBcAg-, and HBx-positive patients had a relatively lower median DFS, but this effect was statistically insignificant (log-rank p > .05) (Table 3). The presence of HBsAg in the tumor, serum, and nontumorous tissue was correlated with shorter DFS and early recurrence compared to HBsAg-negative patients, as shown in Fig. 2, but this effect was not statistically significant (Table 3). Survival analysis for the high-risk group (advanced stages, pT2–4) or higher nuclear grade (ES nuclear grade 3 and 4) showed no significant difference in DFS according to the status of HBV-associated proteins in serum, tumor, and nontumor tissues (data not shown).

### DISCUSSION

In this study, we found that the expression of HBsAg, representing normal viral replication in infected cells, is less frequent in tumors than nontumorous hepatocytes, and HBx, which is one of the key proteins in hepatocarcinogenesis, could be preferentially expressed in different subcellular compartments. The nuclear expression of HBx occurs more frequently in tumors than in nontumorous hepatocytes, whereas the cytoplasmic expression of HBx is less frequent in tumors, according to our results. HCCs with pronounced expression of HBsAg have well-differentiated histology, but HCCs with nuclear expression of HBx showed increased nuclear atypia and aggressive behavior. When the test group is restricted to well-differentiated and to early-stage tumors to minimize the influence of tumor-related prognostic factors, then HCCs expressing nuclear HBX proteins show a higher risk of early recurrence after operation.

Persistence of HBx is important for the pathogenesis of early HCC development, and HBx expression in the liver during chronic HBV infection may be an important prognostic marker for the development of HCC. Even seronegative HBV patients have HBx gene and protein expression in HCC, which are consistent with the hepatocarcinogenic properties of HBx. The subcellular location of HBx, as assessed by immunohistochemistry in previous studies, is generally in the cytoplasm of hepatocytes or tumor cells and is consistent with the biological functions of HBx, i.e., protein-protein interactions in cytoplasmic signaling pathways. The biological role of HBx in the nucleus may involve direct interaction with DNA, RNA, or transcription factors, but the existing data are scarce. Recently, it was shown in an in vitro study using a human cell line that when HBx is targeted to the nucleus by a nuclear localization signal, it can restore HBx-deficient HBV replication, whereas HBx containing a nuclear export signal cannot, suggesting that nuclear localization of HBx is required for viral replication. In addition, chip-based chromatin immunoprecipitation with expression microarray profiling for HCCs identified 184 gene targets that might

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**Table 3.** Disease-free survival analysis of 99 pT1 and well-differentiated HBV-related HCCs in relation to the expression of HBV proteins

| Tumor (n=99) | Recurrence rate  | Median DFS (mo) | p-value |
|-------------|------------------|-----------------|---------|
| HBsAg (-) vs (+) | 38/84 (45.2%) vs 10/15 (66.7%) | 126 vs 35 | .095 |
| HBcAg (-) vs (+) | 44/88 (50.0%) vs 4/11 (36.4%) | 84 vs NA | .619 |
| HBx_nu (-) vs (+) | 20/51 (39.2%) vs 28/48 (58.3%) | 126 vs 35 | .015* |
| HBx_cyto (-) vs (+) | 22/46 (47.8%) vs 26/53 (49.1%) | 126 vs 61 | .541 |

| Nontumorous tissue (n=45) | Recurrence rate  | Median DFS (mo) | p-value |
|---------------------------|------------------|-----------------|---------|
| HBsAg (-) vs (+) | 8/18 (44.4%) vs 16/27 (59.3%) | 135 vs 21 | .379 |
| HBcAg (-) vs (+) | 22/41 (53.7%) vs 2/4 (50.0%) | 135 vs 7 | .805 |
| HBx_nu (-) vs (+) | 17/31 (54.8%) vs 7/14 (50.0%) | 135 vs 33 | .870 |
| HBx_cyto (-) vs (+) | 5/10 (50.0%) vs 19/35 (54.3%) | 33 vs 135 | .837 |

| Serum | Recurrence rate  | Median DFS (mo) | p-value |
|-------|------------------|-----------------|---------|
| HBsAg (n=98) (-) vs (+) | 3/11 (27.3%) vs 44/87 (50.6%) | 126 vs 84 | .296 |
| HBcAb (n=94) (-) vs (+) | 32/74 (43.2%) vs 11/20 (55.0%) | 126 vs 35 | .163 |
| HBcAb (n=57) (-) vs (+) | 2/4 (50.0%) vs 13/33 (39.4%) | 7 vs 84 | .473 |
| HBsAg (n=55) (-) vs (+) | 19/41 (46.3%) vs 6/14 (42.9%) | 84 vs 153 | .505 |
| HBcAb (n=51) (-) vs (+) | 12/21 (57.1%) vs 12/30 (40.0%) | 33 vs 84 | .406 |

**Notes:**
- HBV, hepatitis B virus; HCC, hepatocellular carcinoma; DFS, disease-free survival; HBsAg, HBV surface antigen; HBcAg, HBV core antigen; NA, not applicable; HBx, HBV X protein; nu, nuclear staining; cyto, cytoplasmic staining; HBsAb, anti-HBs; HBcAb, IgG anti-HBc; HBeAg, hepatitis B virus e antigen; HBeAb, anti-HBe.
- *p < .05.
Fig. 2. Cumulative disease-free survival curves of 99 patients with pT1 and well-differentiated hepatocellular carcinoma in relation to the presence of hepatitis B virus (HBV) surface antigen (HBsAg) and HBV X protein (HBx) in a tumor, nontumorous tissue, and serum. HBsAg in tumors (A), HBsAg in nontumorous tissue (B), HBsAg in serum (C), nuclear HBx (HBx nu) in tumors (D), HBX nu in nontumorous tissues (E), and cytoplasmic HBx (HBx cyto) (F) in tumors. *p < .05.
be directly deregulated by HBx via targeting from indirect protein-DNA binding as well as transcriptional factors directly interacting with HBx.17

Our results show that nuclear, not cytoplasmic, HBx expression correlates with aggressiveness of HCC tumors, such as nuclear grade or large vessel invasion, and with early recurrence after an operation. These data suggest that the interaction of HBx with nuclear proteins might be more important for tumor progression than for cytoplasmic interactions of HBx. Although the HBx protein may be involved in metastasis and tumor invasiveness by regulating proteins that control the extracellular matrix, angiogenesis, or epithelial mesenchymal transition, the reason for the paucity of clinical evidence regarding the HBx protein and HCC recurrence or prognosis could be the difficulties with interpretation of HBx immunohistochemistry.18-21 In the majority of the studies on HBx, cytoplasmic staining was considered positive expression, and nuclear staining was not investigated.15,18,19,22 Because we minimized the influence of well-known prognostic factors, such as vascular invasion, size, multiplicity, extent of tumor invasion, and differentiation, by selecting HCCs of pT1 stage and ES nuclear grade 1 and 2, we expected that HBx in nontumorous tissue would be a prognostic factor, but the results showed that it was not. We tried to find a link between the pattern of recurrence and the HBx expression, but the recurrence pattern (presented as a single intrahepatic mass versus multiple or disseminated intrahepatic or extrhepatic masses) was not different depending on the HBx expression in tumor and non-tumor tissues. However, nuclear HBx (−) HCCs both in tumor and nontumor were more frequently presented as a single intrahepatic mass at recurrence time after resection (rate of a single intrahepatic mass, HBx_nu[+]) in tumor and non-tumor vs HBx_nu[+] in tumor or non-tumor 59.3% [16/27] vs 37.0% [27/73], p = .388), but this result was not statistically significant. Further research is needed to understand how nuclear expression of HBx promotes tumor recurrence before pathologic features of tumor aggressiveness have appeared.

The mechanism of HCC recurrence in HBV patients is growth of microscopically or macroscopically leftover tumor cells or de novo occurrence of HCC. The viral influence on HCC recurrence can be explained by the de novo tumor recurrence. Su et al.23 reported that a higher serum HBV DNA load was an important risk factor associated with recurrence in patients with HBV-associated HCC without antiviral therapy after resection, but this observation was not applicable in advanced stage HCC. Tsai et al.16 reported that a ground-glass hepatocyte pattern or HBsAg expression in nontumorous liver tissues was a prognostic marker for the recurrence of HBV-related HCC after hepatic resection; these data also support the viral influence on HCC recurrence. Our finding that HBsAg-expressing HCCs recur earlier than do HBsAg-negative HCCs in patients with early-stage and well-differentiated HCC is in line with the previous reports except for the source of HBsAg production, although this finding was not statistically significant.

Last, our results were limited due to the one core-construct of the tissue microarrays that were used. However, staining on full sections for preliminary study showed that staining pattern was relatively patched, but generally weak to moderate areas were alternatively mixed with strongly stained areas, not totally negative areas, and the size of the study groups (n = 483) might be large enough to compensate this problem.

In summary, we report that nuclear expression of HBx in HBV-associated HCCs increases in tumors compared to nontumorous tissue, and HBsAg-expressing HCCs have a tendency to recur early, even if they are well-differentiated histologically and the surgical procedure is performed at an early stage. Tissue expression of HBsAg was correlated with serum HBsAg. Nuclear expression of HBx in tumor tissue assessed by immunohistochemistry can be a useful prognostic marker for prediction of tumor recurrence in early-stage HCC patients and may ultimately lead to novel therapeutic strategies for managing HBV-associated HCC and for researching HBV-associated hepatocarcinogenesis.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Interobserver Agreement on Pathologic Features of Liver Biopsy Tissue in Patients with Nonalcoholic Fatty Liver Disease

Eun Sun Jung1,2 · Kyoungbun Lee1,3 · Eunsil Yu4 · Yun Kyung Kang1,5 · Mee-Yon Cho6 · Joon Mee Kim7 · Woo Sung Moon8 · Jin Sook Jeong9 · Cheol Keun Park10 · Jae-Bok Park11 · Dae Young Kang12 · Jin Hee Sohn13 · So-Young Jin14

1Gastrointestinal Pathology Study Group of Korean Society of Pathologist; 2Department of Pathology, Seoul St. Mary’s Hospital, College of Medicine, The Catholic University of Korea, Seoul; 3Department of Pathology, Seoul National University College of Medicine, Seoul; 4Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul; 5Department of Pathology, Inje University Seoul Paik Hospital, Seoul; 6Department of Pathology, Yonsei University Wonju College of Medicine, Wonju; 7Department of Pathology, Inha University Hospital, Incheon; 8Department of Pathology, Chonbuk National University Medical School, Jeonju; 9Department of Pathology, Dong-A University College of Medicine, Busan; 10Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul; 11Department of Pathology, Daegu Catholic University College of Medicine, Daegu; 12Department of Pathology, Chungnam National University Hospital, Chungnam National University School of Medicine, Daejeon; 13Department of Pathology, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul; 14Department of Pathology, Soon Chun Hyang University Seoul Hospital, Seoul, Korea

Background: The histomorphologic criteria for the pathological features of liver tissue from patients with non-alcoholic fatty liver disease (NAFLD) remain subjective, causing confusion among pathologists and clinicians. In this report, we studied interobserver agreement of NAFLD pathologic features and analyzed causes of disagreement. Methods: Thirty-one cases of clinicopathologically diagnosed NAFLD from 10 hospitals were selected. One hematoxylin and eosin and one Masson's trichrome-stained virtual slide from each case were blindly reviewed with regard to 12 histological parameters by 13 pathologists in a gastrointestinal study group of the Korean Society of Pathologists. After the first review, we analyzed the causes of disagreement and defined detailed morphological criteria. The glass slides from each case were reviewed a second time after a consensus meeting. The degree of interobserver agreement was determined by multi-rater kappa statistics. Results: Kappa values of the first review ranged from 0.0091–0.7618. Acidophilic bodies (κ = 0.7618) and portal inflammation (κ = 0.5914) showed high levels of agreement, whereas microgranuloma (κ = 0.0984) and microvesicular fatty change (κ = 0.0091) showed low levels of agreement. After the second review, the kappa values of the four major pathological features increased from 0.3830 to 0.5638 for steatosis grade, from 0.1398 to 0.2815 for lobular inflammation, from 0.1923 to 0.3362 for ballooning degeneration, and from 0.3303 to 0.4664 for fibrosis. Conclusions: More detailed histomorphological criteria must be defined for correct diagnosis and high interobserver agreement of NAFLD.

Key Words: Non-alcoholic fatty liver disease; Pathologic features; Interobserver agreement

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Corresponding Author
So-Young Jin, MD
Department of Pathology, Soon Chun Hyang University Seoul Hospital, 59 Daesagwan-no, Yongsan-gu, Seoul 04401, Korea
Tel: +82-2-709-9424
Fax: +82-2-709-9441
E-mail: jin0924@schmc.ac.kr
Nonalcoholic fatty liver disease (NAFLD) is a clinicopathological spectrum characterized by hepatic steatosis without history of significant alcohol use or other known liver disease. The commonly associated factors include obesity, insulin resistance syndrome, and hyperlipidemia. Other associations include jejunooileal bypass/gastroplasty surgery for morbid obesity, parental nutrition, forms of malnutrition, bacterial contamination of the small bowel, certain inherited metabolic disorders, and a wide range of drugs and environmental toxins. The natural history and histological spectrum of NAFLD range from stable simple steatosis to progressive or advanced disease, such as steatohepatitis, cirrhosis, and even hepatocellular carcinoma. A clinicopathological correlation is needed to diagnose NAFLD, as clinical assessment, laboratory tests, and imaging techniques alone provide limited pertinent information. Liver biopsy is the only reliable diagnostic tool to evaluate a patient with suspected NAFLD. Degree of steatosis, liver injury, and fibrosis associated with NAFLD can be identified and differentiated from simple steatosis and nonalcoholic steatohepatitis (NASH), which is the progressive form of NAFLD. A NAFLD histopathological grading system was proposed by Brunt et al. in 1999. They proposed to classify each of steatosis, inflammation, and ballooning degeneration into three grades and fibrosis into four stages. Since then, Kleiner et al. of the NASH Clinical Research Network have proposed a more relevant classification. Other than steatosis, inflammation, and ballooning degeneration, they proposed the classification of fibrosis around the central area into three more detailed grades. Also, they scored each finding and suggested that a score greater than five indicates NASH. However, until now, classifications for NAFLD or NASH have differed depending on the researcher, and no NAFLD histopathological grading system has been standardized, causing confusion for clinicians and pathologists. Furthermore, the histomorphological criteria for NAFLD pathologic features in liver tissue remain subjective. Thus, in this study, we determined the interobserver agreement of NAFLD pathologic features between pathologists and analyzed the causes of the disagreement in order to define the histopathological features in more detail as the basis for a grading system.

MATERIALS AND METHODS

Thirty-one patients with clinically and pathologically diagnosed NAFLD from 10 hospitals (Daegu Catholic University Medical Center, Dong-A University Hospital, Samsung Medical Center, Seoul National University Hospital, Inje University Seoul Paik Hospital, Seoul St. Mary’s Hospital, Soon Chun Hyang University Seoul Hospital, Wonju Severance Christian Hospital, Inha University Hospital, Chungnam National University Hospital) were selected. Selection criteria were clinical NAFLD (non-alcoholic, serologically negative for viral and autoimmune markers, abnormal levels of liver enzymes such as asparaginaminotransferase and alanine aminotransferase) and age ≥ 19 years. Cirrhosis cases were included, and cases of drug and toxic injury conditions were excluded. Fifty-one liver biopsies from 10 hospitals were collected. Among them, 31 biopsies (≥ 1.5 cm in length and ≥ 16 G needle size) were selected. One hematoxylin and eosin (H&E)- and one Masson’s trichrome–stained slide were selected from each of the 31 cases. The biopsy specimens were anonymized and randomized by a researcher not involved in the study. All selected slides were scanned by a virtual slide scanning system (3DHistotech Ltd., Budapest, Hungary) at Asan Medical Center in Seoul.

The following 12 NAFLD pathologic features were selected: steatosis grade, steatosis location, microvesicular steatosis, fibrosis stage, lobular inflammation, microgranuloma, large lipogranuloma, portal inflammation, ballooning degeneration, acidophilic bodies, Mallory’s hyaline, and glycogenated nuclei. Each parameter was reviewed and scored using the detailed scoring criteria shown in Table 1.

One H&E- and one Masson’s trichrome–stained virtual slide from each case were reviewed for the 12 parameters. Reviews were performed blindly by 13 pathologists from a gastrointestinal study group of the Korean Society of Pathologists. The degree of interobserver agreement for the first review was analyzed by multi-rater Kappa statistics.

The results were shared with all 13 pathologists, and a consensus meeting was held after the first review to analyze the reasons for disagreement and to define the morphologic criteria in more detail.

After the consensus meeting, a second review of the 12 pathological parameters was performed using glass slides from each of the 31 cases. The degree of interobserver agreement after the second review was analyzed by multi-rater Kappa statistics and compared with the results of the first review.

The Institutional Review Board of Seoul St. Mary’s Hospital approved this study (KIRB-00562_5-001).

RESULTS

Kappa values of interobserver agreement for the first review ranged from 0.0091 to 0.7618 (Table 2). The order of agreement,
according to the kappa value, was acidophilic bodies (k = 0.7618), portal inflammation (k = 0.5914), large lipogranuloma (k = 0.4822), Mallory's hyaline (k = 0.4603), steatosis grade (k = 0.3830), steatosis location (k = 0.3388), glycogenated nuclei (0.3218), ballooning degeneration (k = 0.1923), lobular inflammation (0.1398), microgranuloma (0.0984), and microvesicular fatty change (0.0091). The kappa values of the four major pathologic features (steatosis grade, portal inflammation, ballooning degeneration, and fibrosis) were measured as 0.3829, 0.5913, 0.1923, and 0.3303, respectively. In particular, ballooning degeneration (k = 0.1923), which is an important feature for diagnosis of NASH, showed a low level of agreement.

Kappa values of interobserver agreement for the second review ranged from 0.1199 to 0.7386 (Table 2). The order of kappa values for interobserver agreement after the second review were portal inflammation (k = 0.7386), acidophilic bodies (k = 0.6493), steatosis grade (k = 0.5638), Mallory's hyaline (k = 0.5236), large lipogranuloma (k = 0.5004), fibrosis (k = 0.4664), steatosis location (k = 0.4502), glycogenated nuclei (k = 0.3846), ballooning degeneration (k = 0.3362), microvesicular fatty change (k = 0.2916), lobular inflammation (k = 0.2815), and microgranuloma (k = 0.1199). The kappa values of interobserver agreement increased for all parameters except acidophilic bodies. Microvesicular steatosis demonstrated the largest improvement (k = 0.0091 to 0.2916), and microgranuloma the smallest (k = 0.0984 to 0.1199). All kappa values of the four major pathological features increased as follows: steatosis grade from k = 0.3830 to 0.5638, portal inflammation from k = 0.5914 to 0.7386, ballooning degeneration from k = 0.1923 to 0.3362, and fibrosis from k = 0.3303 to 0.4664.

**DISCUSSION**

Since the first description of NASH by Ludwig et al. in 1980,18 several NAFLD scoring schemes have been proposed.16,17,19,20 Among them, the NAFLD activity score (NAS) proposed by Kleiner et al.17 is the most well-known and popular system. Their

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**Table 1.** Pathological parameters and detailed scoring criteria of non-alcoholic fatty liver disease

| Pathologic parameter | Criteria for scoring |
|----------------------|----------------------|
| 1. Steatosis grade   | 1: <5%, 2: 5–33%, 3: 34–66%, 4: >66% |
| 2. Steatosis location| 1: zone 1, 2: zone 3, 3: azonal, 4: panacinar |
| 3. Microvesicular steatosis | 0: absent, 1: present |
| 4. Fibrosis | 0: none |
| 5. Lobular inflammation | 1: <2 foci per 200 × field |
| 6. Microgranuloma | 0: absent, 1: present |
| 7. Large lipogranuloma | 0: absent, 1: present |
| 8. Portal inflammation | 0: none to minimal, 1: greater than minimal |
| 9. Ballooning | 0: absent, 1: few, 2: many/prominent |
| 10. Acidophilic bodies | 0: none to rare, 1: many |
| 11. Mallory's hyaline | 0: none to rare, 1: many |
| 12. Glycogenated nuclei | 0: none to rare, 1: many |

**Table 2.** Comparison of kappa values after the first and second reviews of non-alcoholic fatty liver disease pathological features

| Pathologic parameters | First review | Second review |
|-----------------------|-------------|--------------|
| 1. Steatosis grade     | 0.3830      | 0.5638       |
| 2. Steatosis location  | 0.3388      | 0.4502       |
| 3. Microvesicular steatosis | 0.0091    | 0.2916       |
| 4. Fibrosis            | 0.3303      | 0.4664       |
| 5. Lobular inflammation | 0.1398      | 0.2815       |
| 6. Microgranuloma      | 0.0984      | 0.1199       |
| 7. Large lipogranuloma | 0.4822      | 0.5004       |
| 8. Portal inflammation | 0.5914      | 0.7386       |
| 9. Ballooning          | 0.1923      | 0.3362       |
| 10. Acidophilic bodies | 0.7618      | 0.6493       |
| 11. Mallory's hyaline  | 0.4603      | 0.5236       |
| 12. Glycogenated nuclei | 0.3218     | 0.3846       |
proposed NAS system is based on agreement data and a multiple regression analysis of the 14 histological features of steatosis grade, steatosis location, microvesicular steatosis, fibrosis, lobular inflammation, microgranuloma, large lipogranuloma, portal inflammation, ballooning degeneration, acidophilic bodies, pigmented macrophages, megamitochondria, Mallory’s hyaline, and glycogenated nuclei. The NAS is defined as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning (0–2) and ranges from 0 to 8. Fibrosis was not included as an NAS component. Kleiner et al. reported that the interobserver agreement values for the four major features were 0.79 for steatosis grade, 0.45 for lobular inflammation, 0.56 for ballooning degeneration, and 0.84 for fibrosis. The agreement for other histologic features ranged from \( k = 0.15 \) to 0.58. However, the histomorphological features of some parameters remain ambiguous, contributing to low interobserver agreement. We studied interobserver agreement among 13 pathologists for each of the 12 well-known parameters and analyzed the reasons for disagreement. At the first circulation of slides, we reviewed each case without consensus to identify current discrepancies in diagnostic criteria. The kappa values in this review ranged widely from 0.0091 to 0.7618. Kappa values of the four major pathological features at the first review were 0.3830 for steatosis grade, 0.1398 for lobular inflammation, 0.1923 for ballooning degeneration, and 0.3303 for fibrosis, lower than those of Kleiner et al.

After the first review, we discussed several points of debate surrounding the definition of each parameter. As a result, we identified several details regarding steatosis grade, lobular inflammation, ballooning degeneration, fibrosis, Mallory’s body, and microvesicular fatty change and recommend the following:

1. Steatosis grade: steatosis grade should be determined by fat volume rather than the number of fatty hepatocytes at 100× optical magnification (Fig. 1).

2. Lobular inflammation: lobular inflammation should be graded under 200× magnification throughout the entire biopsy field, and the mean, not the maximum number in the most active field, should be determined. Spotty necrosis of hepatocytes, lymphocyte aggregations, and acidophilic bodies should be included, whereas lipogranuloma resulting from fat phagocytosis should be excluded as in the histomorphologic criteria for chronic hepatitis grading (Fig. 2).

3. Ballooning degeneration: the histomorphological criteria of ballooning degeneration are enlarged round cells with loss of

Fig. 1. Grading of steatosis. It is determined by fat volume rather than number of fatty hepatocytes. (A) <5%. (B) 5%–33%. (C) 34%–66%. (D) > 66%.
polygonal features and cytoplasm showing heterogeneous granular features (Fig. 3). Hydropic swelling and microvesicular fatty changes should be carefully distinguished from ballooning degeneration. In cases of hydropic swelling, the hepatocyte has large, swollen, and homogenously granular cytoplasm with well-preserved polygonal features. In particular, microvesicular fatty changes can also be enlarged and can be confused with ballooning degeneration. Microvesicular fatty changes show centrally located nuclei indented by a small fat droplet with a lipoblast-like feature (Fig. 4). When only one or two ballooned cells can be seen throughout the entire field, the term “few” can be applied.

(4) Fibrosis: mild fibrosis should be carefully distinguished from the normal framework around the central area. Only obvious fibrosis with pericellular collagen deposition should be considered as existence of fibrosis (Fig. 5).

(5) Mallory’s body: Mallory’s body should be defined as a definite eosinophilic lump in the cytoplasm (Fig. 3B).

(6) Microvesicular fatty changes: these are defined as lipoblast-like features showing centrally located nucleus and numerous intracytoplasmic micro-fat vacuoles inducing nuclear indentation. Microvesicular fatty changes should be carefully differentiated from cells having small- or medium-sized fat vacuoles without cytoplasmic enlargement and nuclear indentation, as mentioned by Yeh and Brunt (Fig. 6).

All kappa values increased in the second review based on the above criteria. In particular, kappa values for the four major parameters increased from 0.3830 to 0.5638 for steatosis grade, from 0.1398 to 0.2815 for lobular inflammation, from 0.1923 to 0.3362 for ballooning degeneration, and from 0.3303 to 0.4664 for fibrosis.

This increased agreement likely resulted from the consensus
meeting and the determination of more detailed histomorphologic criteria. However, the method used in the second review is more familiar to most pathologists and may have also contributed to increased agreement. Despite the differences in review method (virtual vs glass), there is no doubt that the exact histomorphologic criteria of NAFLD remain ambiguous and con-

**Fig. 4.** Mimickers of ballooning degeneration. (A, B) Hydropic swelling and microvesicular fatty changes mimicking ballooning degeneration.

**Fig. 5.** Fibrosis around the central area (A), normal framework (B), normal range framework without pericellular collagen deposition (C), and mild perisinusoidal fibrosis with definite pericellular collagen deposition.

**Fig. 6.** Microvesicular steatosis. (A) Microvesicular fatty changes including lipoblast-like features showing numerous intracytoplasmic micro-fat vacuoles with nuclear indentation (arrows). (B) Excluding small- or medium-sized fat vacuoles without cytoplasmic enlargement and nuclear indentation should not be confused with microvesicular steatosis.
tribute to low interobserver agreement between pathologists. Therefore, our more detailed suggestions for NAFLD histomorphologic criteria—including steatosis grade, lobular inflammation, ballooning degeneration, and fibrosis, as mentioned above—will increase the accuracy of diagnosis and grading of NAFLD and improve interobserver agreement. Through this work and recommendations, we expect that a more exact basis for research of NAFLD and development of a new grading and scoring system will follow.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Non-small Cell Lung Cancer with Concomitant \textit{EGFR}, \textit{KRAS}, and \textit{ALK} Mutation: Clinicopathologic Features of 12 Cases

Taebum Lee · Boram Lee
Yoon-La Choi · Joungho Han
Myung-Ju Ahn · Sang-Won Um

Department of Pathology, 1Division of Hematology-
Oncology, Department of Medicine, 2Division of
Pulmonary and Critical Care Medicine,
Department of Medicine, Samsung Medical
Center, Sungkyunkwan University School of
Medicine, Seoul, Korea

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Corresponding Author
Joungho Han, MD
Department of Pathology, Samsung Medical Center,
Sungkyunkwan University School of Medicine,
81 Innon-ro, Gangnam-gu, Seoul 06351, Korea
Tel: +82-2-3410-2800
Fax: +82-2-3410-0025
E-mail: hanjho@skku.edu

Background: Although epidermal growth factor receptor (\textit{EGFR}), v-Ki-ras2 Kirsten rat sarcoma viral oncogene (\textit{KRAS}), and anaplastic lymphoma kinase (\textit{ALK}) mutations in non-small cell lung cancer (NSCLC) are the most commonly mutated oncogenes that involve the pathogenesis of lung cancer as “genetic drivers.” Advanced NSCLC has a dismal prognosis with a short median overall survival, but several selective \textit{EGFR} tyrosine kinase inhibitors (TKIs) and an \textit{ALK} inhibitor show effectiveness as personalized target therapy in patients who harbor those specific genetic mutations. According to guidelines from the College of American Pathologists, the International Association for the Study of Lung Cancer (IASLC) and the Association for Molecular Pathology, mutation analysis is recommended and recent studies have demonstrated the cost-effectiveness of genetic screening, especially in Asians, who have a higher prevalence of \textit{EGFR} mutations.

Chromosomal alterations, closely related to unique histologic phenotypes through protein expression, determine differentiation, patterns, and cell types. The identification of typical morphological features of certain mutations allows retrospective speculation on underlying genetic alterations. Although \textit{EGFR}, \textit{KRAS}, and \textit{ALK} mutations have generally been considered mutually exclusive in NSCLCs, some cases harbor double mutations in a single tumor. Identifying driver oncogenes in double-mutated tumors through morphologic differences and comparing the responsiveness of targeted therapies can help explain the pathogenesis of lung cancer and predict resistance to targeted therapy. Hence, we explored histologic features and genetic drivers of tumors with concomitant mutations and discovered the dominant mutation in carcinogenesis for each.

MATERIALS AND METHODS

A retrospective review was performed of biopsied and/or sur-
gically resected cases diagnosed as NSCLC and tested for \textit{EGFR}, 
\textit{KRAS}, and \textit{ALK} mutations in the Department of Pathology and 
Translational Genomics at Samsung Medical Center, Seoul, Korea from 2006 to 2014. Of 6,637 NSCLC patients, 6,595 \textit{EGFR}, 
5,177 \textit{KRAS}, and 4,869 \textit{ALK} mutation tests were performed. 
Among them, 2,387 patients (36.2\%) had \textit{EGFR}, 398 (7.7\%) had 
\textit{KRAS}, and 281 (5.8\%) had \textit{ALK} mutations. Based on these 
tests, we selected 12 patients with concomitant mutations in the 
same tumor.

Clinical data, including gender, age, smoking history, and previous concurrent chemoradiation therapy history were extracted from electronic medical records. All hematoxylin and eosin stained slides of selected cases were reviewed by two pathologists (T.L. and B.L.). Histologic classification was made according to the IASLC/American Thoracic Society (ATS)/European Respiratory Society (ERS) International Multidisciplinary Classification of Lung Adenocarcinoma, and typical pathologic features were re-evaluated, including cell type, nuclear and cytoplasmic characteristics.

We used two methods to evaluate \textit{EGFR} mutations in the 18th, 19th, 20th, and 21st exon: Sanger sequencing and real time polymerase chain reaction after peptide nucleic acid (PNA)–clamping using the PNA clamping \textit{EGFR} Mutation Detection Kit (Panagene, Inc., Daejeon, Korea). \textit{KRAS} mutations were evaluated with Sanger sequencing in the 12th and 13th exon. Extracted genomic DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue was used for \textit{EGFR} and \textit{KRAS} analyses. \textit{ALK} mutation tests were performed using immunohistochemical (IHC) (1:40, NCL-ALK, clone 5A4, Novocastra, Newcastle upon Tyne, UK) and \textit{fluorescence in situ} hybridization (FISH) analysis (LSI ALK dual color break-apart probe, Dako, Glostrup, Denmark) with FFPE tissue. Diffuse strong positivity in the cytoplasm was regarded as positive in ALK IHC. In the FISH analysis, 50 non-overlapping nuclei were counted and 15\% of break-apart probes were used as a cutoff value for positivity. Diffuse strong positive ALK IHC results were interpreted as a surrogate marker of \textit{ALK} rearrangement.\cite{14}

**RESULTS**

**Clinical characteristics**

Clinical data and pathologic features of the 12 patients with concomitant mutations are summarized in Table 1. The age at diagnosis of patients ranged from 48 to 78 years old (median, 62 years old) and 75\% of patients were female. All three male patients had a history of smoking (15–57 pack-years) in contrast to none of female patients. Five surgically resectable patients underwent lobectomy and seven clinical stage IV patients had a needle aspiration or biopsy from the lung, bronchus, lymph node, or adrenal gland.

Patient No. 5 received \textit{EGFR} TKI targeted therapy for bone metastasis at 15 months after lobectomy. Patient No. 6 refused treatment with chemo- or targeted therapy and patient No. 7 had a history of lobectomy at an outside hospital without any other treatment 9 years prior to the lymph node biopsy. Patient No. 10 received neoadjuvant concurrent chemoradiation therapy prior to surgery. Patient No. 12 previously underwent lobectomy and was treated with gefitinib for 1 month and crizotinib for 4 months in China. She had no \textit{EGFR} or \textit{KRAS} mutation at the time of diagnosis at China. Aside from this patient, all other patients had no history of prior targeted therapy before mutation testing.

**Mutational and histologic characteristics**

All 12 patients were diagnosed with adenocarcinoma and had an \textit{EGFR} mutation. \textit{EGFR} mutations of three patients were only detected with the PNA clamping method, while nine were confirmed by Sanger sequencing. Five (41.7\%) had the missense L858R mutation in exon 21, four (33.3\%) had the missense G719X mutation in exon 18, and three (25\%) had a deletion in exon 19. Patient No. 8 had the L858R and G719X mutations at the same time and patient No. 12 had a missense R803W mutation at exon 20 that was not identified before targeted therapy at the outside hospital. No patient had a T790M point mutation, which is associated with resistance. Six patients had additional \textit{KRAS} mutations and the other six had an \textit{ALK} mutation (referred to as \textit{’EGFR-KRAS’} and \textit{’EGFR-ALK’} hereafter). Three among the six patients who showed positivity at ALK IHC were confirmed with FISH analysis.

\textit{EGFR-KRAS} and \textit{EGFR-ALK} tumors showed distinct morphologic characteristics. Six \textit{EGFR-KRAS} patients had papillary, acinar, solid, and micropapillary patterns (Fig. 1). Among them, five patients had typical hobnail cell features with apical snouts; the remaining patient (patient No. 2) underwent needle aspiration and did not show any typical cell features. One patient (patient No. 4) had a focal columnar cell component and intra- and extracytoplasmic mucin, which are similar morphologic features of NSCLC with a \textit{KRAS} mutation. The other five patients did not show any mucin. Two of the six patients had intranuclear inclusions.

In contrast, six \textit{EGFR-ALK} patients showed solid, cribriform and micropapillary patterns. Three of six patients had signet ring
Table 1. Clinicopathologic features of 12 patients with double mutated pulmonary adenocarcinoma

| Patient No. | Sex/ Age (yr) | Smoking | Specimen | Stage   | EGFR mutation | Additional mutation | Dominant pattern | Typical cell feature | Mucin | Intranuclear inclusion | Surgery | Targeted therapy | Response | Status | PFS (mo) |
|-------------|---------------|---------|----------|---------|---------------|---------------------|------------------|----------------------|-------|----------------------|----------|---------------------|----------|--------|---------|
| 1           | M/74          | 57PY    | Lung/R   | pT1bN1  | E21 L858R     | KRAS G12S         | Papillary and micropapillary | Hobnail cells | No | No                   | +        | -                  | -        | DOD a   | -       |
| 2           | F/62          | Never   | Lung/A   | M1(M)   | E21 L858R     | KRAS G12D         | No               | No                  | Present | Gefitinib            | PR → PD  | DOD                | 7        |        |         |
| 3           | F/63          | Never   | Lung/R   | pT1bN0  | E21 L858R     | KRAS G13A         | Papillary and acinar | Hobnail cells | No | No                   | +        | -                  | -        | NED     | -       |
| 4           | F/48          | Never   | Lung/R   | pT1bN2  | E19 deletion  | KRAS G12V         | Acinar and solid    | Hobnail and columnar cells | Intra- and extracytoplasmic | No   | +                   | Erlotinib | PR → PD  | DOD                | 20  |
| 5           | M/55          | 20PY    | Lung/R   | pT2N2   | E19 deletion  | KRAS G13C         | Solid and acinar    | Hobnail cells | No | Present              | +        | Gefitinib          | PR       | AWD     | 29      |
| 6           | F/78          | Never   | Lung/B   | M1(M)   | E18 G719X     | KRAS G12D         | Papillary and acinar | Hobnail cells | No | No                   | -        | (refused)          | -        | AWD a   | -       |
| 7           | F/62          | Never   | LN/B     | M1(M)   | E21 L858R     | ALK                | Solid and cribriform | Signet ring cells | Intracytoplasmic | No   | + d | Gefitinib          | PR       | AWD a   | 18 e   |
| 8           | F/62          | Never   | Lung/B   | M1(M)   | E21 L858R     | ALK                | Solid and cribriform | No                  | No | No                   | -        | Gefitinib and Crizotinib | SD and PR | AWD     | 24      |
| 9           | M/56          | 15PY    | Lung/B   | M1(M)   | E18 G719X     | ALK                | Solid              | No                  | No | No                   | -        | Crizotinib         | SD       | DOD     | 4       |
| 10          | F/68          | Never   | Lung/R   | ypT2N2  | E18 G719X     | ALK                | Solid, micropapillary and cribriform | Signet ring cells | Intracytoplasmic | Present | +   | -                  | -        | NED     | -       |
| 11          | F/58          | Never   | Lung/B   | M1(M)   | E19 deletion  | ALK                | Solid              | Signet ring cells | Intracytoplasmic | No     | -      | Crizotinib | -        | AWD a | -       |
| 12          | F/66          | Never   | Adrenal/B | M1(M)   | E20 R803W     | ALK                | Solid              | No                  | No | No                   | + d | Erlotinib          | PD       | AWD a | 0.7     |

EGFR, epidermal growth factor receptor; PFS, progression-free survival; M, male; PY, pack-year; R, resection; E, exon; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene; DOD, died of disease; F, female; A, aspiration; PR, partial response; PD, progressive disease; NED, no evidence of disease; AWD, alive with disease; B, biopsy; LN, lymph node; ALK, anaplastic lymphoma kinase; SD, stable disease.

aLost to follow-up; bCannot identify dominant architectural patterns in an aspiration slide; cMutation detected only in PNA clamping method; dHave a history of lobectomy at outside hospital, prior to biopsy of metastatic lesion; eCannot evaluate due to lost to follow-up.
cells and intracytoplasmic mucin with or without extracytoplasmic mucin production, which are typical features of ALK-positive NSCLC. The others did not show any typical cell features or mucins. One patient had intranuclear inclusions.

**Treatment responses and follow-up status**

Seven patients had surgical resection and two were alive without targeted therapy and any evidence of relapse or progression, including one patient who received neoadjuvant concurrent chemoradiation therapy. One patient who had surgery and de-
cided not to receive targeted therapy due to old age and underlying diabetes mellitus died of disease after 53 months from surgery (patient No. 1).

EGFR TKI included gefitinib (250 mg, orally, every day) and erlotinib (150 mg, orally, every day) and the ALK inhibitor was crizotinib (250 mg, orally, every day or twice daily). Three among six EGFR-KRAS patients received targeted therapy with EGFR TKI. All three patients showed partial responses, but two also showed disease progression after 7 and 20 months. In the EGFR-ALK patients, two were treated with EGFR TKI, two with ALK inhibitor and one received both EGFR TKI and ALK inhibitor therapy. Most patients treated with ALK inhibitor or EGFR TKI showed partial response or stable disease, but patient No. 12 with EGFR TKI showed progressive disease.

DISCUSSION

Concomitant genetic alteration of NSCLC is unusual because EGFR, KRAS, and ALK mutations are widely known as mutually exclusive. Most are associated with acquired mutation after targeted therapy and are related to drug resistance. In previous studies, two different hypotheses were proposed to explain dual mutation in NSCLC. Underlying intratumoral heterogeneity of EGFR mutations, different tumor cells may have different oncogenic driver mutations. Some authors have also suggested coexistence of mutations in the same tumor cells using IHC expression and mutation-specific antibodies.

EGFR, as a growth factor membrane-bound receptor tyrosine kinase, controls cell proliferation and survival. Approximately 36% of patients with NSCLC in East Asia have EGFR mutations and 90% of mutations are exon 21 L858R or exon 19 deletion. These mutations increase tyrosine kinase activity, resulting in hyperactivation of the RAS-RAF-MEK-ERK pathway, which regulates cell proliferation, and the phosphomonoesterase 3-kinase–AKT–mammalian target of rapamycin pathway, which regulates cell proliferation, and the phosphoinositide 3-kinase–AKT pathway.

In our study, however, three patients showed partial response to gefitinib and erlotinib for 7–29 months. Intratumoral heterogeneity of the mutation may explain this discordance between coexistent KRAS mutation and response to EGFR TKI therapy. Patient No. 2 had short progression-free survival (PFS) and disease progressed rapidly, while the other two patients had 20–29 months of PFS. Based on the evidence of morphologic phenotypes and responsiveness to targeted therapy, we expected these two patients to have coexistent mutations in the same tumor cells, and hypothesized that the EGFR mutation was the dominant oncogene in lung cancer, even if the tumor cells harbored an additional KRAS mutation.

In EGFR-ALK patients, some previous studies have shown acquired EGFR mutations as a mechanism of resistance to ALK inhibitor therapy. In our case, patient No. 12 had no EGFR or KRAS mutation and was treated with crizotinib in an outside hospital. After 4 months of ALK-targeted therapy, she acquired an additional EGFR mutation and showed poor response to erlotinib, which is consistent with previous studies. In the other four patients, however, diverse responses were identified with no resistance to gefitinib or crizotinib. Conflicting results of responsiveness to EGFR TKI and ALK inhibitor in EGFR-ALK patients in NSCLC have been reported in a few limited studies. Recently, Yang et al. investigated relationships between receptor phosphorylation and response to targeted therapy, and suggested that relative phospho-EGFR and ALK levels can be used to predict response.

Although the G719X mutation accounts for about 7% of
EGFR mutated NSCLC in East Asia,\textsuperscript{17} three of five EGFR-ALK patients had the EGFR exon 18 G719X missense mutation in our study. In addition, three of five EGFR-ALK patients showed typical histologic features of ALK-expressing adenocarcinoma. From these findings, we suspect that the ALK mutation determines morphological phenotypes and acts as a driver oncogene, and the EGFR mutation may also play an important role in oncogenesis. Accordingly, based on evaluation of the signaling pathway, including phosphorylation of receptor kinase, the modification or combination of EGFR TKI and ALK inhibitor offer promising treatments.

The small number of cases limited our analysis. Baldi et al.\textsuperscript{18} revealed the possibility of more frequent concomitant mutations than expected and Won et al.\textsuperscript{19} reported an increased proportion of NSCLC harboring concomitant EGFR and ALK mutations from 4.4% to 15.4% using more sensitive methods such as targeted next-generation sequencing (NGS) and mutant-enriched NGS. Greater numbers of previously undisclosed concomitantly mutated tumors can be identified using more sensitive and advanced techniques. Some authors also suggest that tumor burden of each mutation affects responses to targeted therapy. There may be a limitation to evaluating mutations from biopsy specimens, but the majority of patients who need to receive targeted therapy might be clinically unresectable and may only be diagnosed with biopsy. Thus, the expectation of tumor burden in a limited biopsy specimen is unfavorable.

To the best of our knowledge, this is the first study to describe double mutated NSCLC with histologic findings. Morphologic findings might help predict underlying driver mutations and further studies are warranted.

In conclusion, we investigated genetic driver mutations in 12 double mutated NSCLCs with analysis of clinical and pathologic features. The majority of EGFR-KRAS tumors showed typical histologic patterns and cell features of EGFR-mutated tumors and partially responded to EGFR TKI. In EGFR-ALK tumors, however, some showed ALK-mutated features and partially responded to ALK inhibitor or EGFR TKI. EGFR and ALK play an important role in the oncogenesis of NSCLC and tumor morphology can provide important clues to predict treatment response. We suggest that patients with histologic features of EGFR mutations can be treated with EGFR TKI, even with coexistence of a KRAS mutation. We also suggest that EGFR-ALK patients should have underlying mutational pathways evaluated and be treated with a selection and/or combination of ALK inhibitor and EGFR TKI. Further studies are needed to support these interesting findings.

Conflicts of Interest
No potential conflict of interest relevant to this article was reported.

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Analysis of Surgical Pathology Data in the HIRA Database: Emphasis on Current Status and Endoscopic Submucosal Dissection Specimens

Sun-ju Byeon · Woo Ho Kim

Department of Pathology, Seoul National University Hospital, Seoul, Korea

Background: In Korea, medical institutions make claims for insurance reimbursement to the Health Insurance Review and Assessment Service (HIRA). Thus, HIRA databases reflect the general medical services that are provided in Korea. We conducted two pathology-related studies using a HIRA national patient sample (NPS) data (selection probability, 0.03). First, we evaluated the current status of general pathologic examination in Korea. Second, we evaluated pathologic issues associated with endoscopic submucosal dissection (ESD).

Methods: The sample data used in this study was HIRA-NPS-2013-0094. In the NPS dataset, 163,372 pathologic examinations were performed in 103,528 patients during the year 2013. Considering sampling weight (33.3), it is estimated that 5,440,288 (163,372 x 33.3) pathologic examinations were performed. Internal medicine and general surgery were the most common departments requesting pathologic examinations. The region performing pathologic examinations were different according to type of medical institution. In total, 490 patients underwent ESD, and 43.4% (213/490) underwent ESD due to gastric carcinoma. The results of the ESD led to a change in disease code for 10.5% (29/277) of non-gastric carcinoma patients. In addition, 21 patients (4.3%) underwent surgery following the ESD. The average period between ESD and surgery was 44 days.

Conclusions: HIRA sample data provide the nation-wide landscape of specific procedure. However, in order to reduce the statistical error, further studies using entire HIRA data are needed.

Key Words: Statistics; Sample size; Pathology; Surgery

In Korea, all medical institutions claim insurance reimbursements for services to the Health Insurance Review and Assessment Service (HIRA). Thus, HIRA databases reflect the general medical services that are provided in Korea. Since the entire HIRA database is too big to analyze, HIRA provides a specific set of data according to researcher's requests. HIRA also provides some relatively small sized data sets (statistically extracted, anonymized, and annualized) for research and public purposes. These sample data included national patient sample (NPS), a national inpatient sample, an adult patient sample (65 years or older), and a pediatric patient sample. To the best of our knowledge, this is the first pathology-related analysis using HIRA data.

We conducted two pathology-related studies using HIRA-NPS data. First, we analyzed the rate of surgical pathologic examinations in Korea. After Kamegoro Inamoto introduced pathology to Korea in 1914, pathologic examinations have played a major role in medical services for the improvement of overall care. However, research on the frequency of pathologic examinations in this nation as a whole have not been performed due to a restriction on the sharing of personal medical records.

Next, we analyzed the pathologic results of endoscopic submucosal dissection (ESD) specimens. ESD is considered as an initial treatment modality for early and localized gastric carcinoma or benign epithelial neoplasm. We evaluated several ESD-related parameters. After ESD and pathologic examination, surgical treatment is called for in those cases that show tumor cells in the resection margins, endolymphatic tumor emboli, or submucosal invasion. Several papers have been published on this subject, but most of them are from a single institute and so do not incorporate the data of those patients who later went on to undergo additional surgery in different hospitals. One of the advantageous features of the HIRA database is that patients can be tracked through several medical institutions, thereby enabling the study of a more complete data set including those that received secondary treatment in one hospital after ESD in another hospital.

MATERIALS AND METHODS

The sample data used in this study was HIRA-NPS-2013-0094.
This sample was composed of 26 text files, and the total file size was 16.6 gigabytes (GB). The HIRA data tables were composed of five main tables (general specification, healthcare services, diagnosis information, prescriptions, and general information about the medical institutions). Each table was conjoined with proper claim numbers or medical institution numbers. The disease codes were based on the Korean Standard Classification of Disease (KCD). Specifications of the computer that was used to analyze the data were as follows: central processing unit (CPU), i3-2120 3.30 GHz (Intel, Santa Clara, CA, USA); 32 GB main memory; operating system (OS), Ubuntu 14.04.3 long-term support (LTS); and R 3.2.2 analysis software. The pathologic examination codes used in this study are summarized in Table 1. The sample data contained 22,344,536 claims in 1,361,717 patients (selection probability, 0.03; sampling weight, 33.3).

| Table 1. Pathologic examination claim codes using in this study |
|---------------------------------------------------------------|
| | Code |
| Biopsy: 1–3 pieces/4–6 pieces/7–9 pieces/10–12 pieces/more than 13 pieces | C5911/C5912/C5913/C5914/C5915 |
| Resected specimen requiring gross sectioning (non-malignant): paraffin blocks ≤6/paraffin blocks ≥7 | C5916/C5917 |
| Resected specimen for malignant tumor requiring gross sectioning | |
| With lymph node dissection: paraffin blocks ≤20/paraffin blocks ≥21 | C5918/C5919 |
| Without lymph node dissection: paraffin blocks ≤15/paraffin blocks ≥16 | C5500/C5504 |
| Histologic mapping of tumor: with lymph node dissection/without lymph node dissection | C5505/C5506 |
| Emergency histopathologic examination during surgery (frozen section): 1–2 specimens/3–6 specimens/more than 7 specimens | C5511/C5512/C5513 |
| Tissue immunofluorescent microscopic examination: IgG/IgA/IgM/IgE/C3/C4/HbsAg/fibrinogen/others | C5541/C5542/C5543/C5544/C5545/C5546/C5547/C5548 |
| Tissue electron microscopy | C5550 |
| Enzyme histochemistry: ATPase-pH 9.4/ATPase-pH 4.9/NADH/acetylcholinesterase/chloroaacetate esterase/others | C5561/C5562/C5563/C5564/C5565/C5566 |
| Immunohisto(cyto)chemistry | C5575/C5575006 |
| Cervicovaginal smear/Liquid-based cervicovaginal cytology | C5920/CX541 |
| Body fluid: general/cytospin/cell block after cytopathologic examination/liquid-based body fluid cytopathology | C5930/C5931/C5940/CZ521 |
| Aspiration/Aspiration and cell block | C5941/C5942 |
| Liquid-based aspiration cytology/Liquid-based aspiration cytopathology and Cell block | C5943/C5944 |
| Fluorescence in situ hybridization: HER2 gene | C5967/C5967006 |
| Silver in situ hybridization: HER2 gene | C2968/C2968006 |

HBSAg, hepatitis B surface antigen.

| Table 2. Pathologic examinations claims and requesting medical institutions according to administrative districts (sort based on the total number of claims) |
|---------------------------------------------------------------|
| Administrative district | Tertiary hospital | General hospital | Other institutions | Total | Total estimates |
| Seoul | 25,547 | 16,164 | 12,000 | 53,711 | 1,788,576 |
| Gyeonggi-do | 1,632 | 16,203 | 10,906 | 28,740 | 957,042 |
| Busan | 3,276 | 4,877 | 5,135 | 13,288 | 442,490 |
| Daejeju | 2,545 | 2,377 | 2,465 | 7,387 | 245,987 |
| Gyeongsangnam-do | 760 | 3,561 | 3,053 | 7,374 | 245,554 |
| Gwangju | 1,211 | 2,438 | 1,910 | 5,559 | 185,115 |
| Daejeon | 970 | 2,300 | 1,781 | 5,051 | 168,198 |
| Jeollabuk-do | 857 | 1,305 | 2,162 | 4,954 | 164,968 |
| Gyeongsangbuk-do | - | 2,252 | 2,638 | 4,890 | 162,837 |
| Jeollanam-do | - | 2,655 | 1,621 | 4,276 | 142,391 |
| Chungcheongnam-do | - | 2,024 | 1,861 | 3,885 | 129,371 |
| Gangwon-do | 1,179 | 1,046 | 3,687 | 122,777 |
| Gangwonbuk-do | 573 | 1,178 | 1,611 | 3,362 | 111,955 |
| Ulsan | - | 1,561 | 1,799 | 3,360 | 111,888 |
| Jeju | - | 1,135 | 380 | 1,515 | 50,450 |
| Sejong-si | - | - | 51 | 51 | 1,698 |
| Total | 39,385 | 68,384 | 55,603 | 163,372 | 5,440,288 |
| Department code | Biopsy | Resection, non-malignant | Resection, malignant (LN/non-LN) | Histologic mapping (LN/non-LN) | Frozen sections | IF | EM | Enzyme | IHC | Uterine cervical examinations (smear/liquid) | Body fluid (conventional/liquid) | Aspiration (conventional/liquid) | HER2 (FISH/SISH) | Total | Total estimates |
|----------------|--------|-------------------------|---------------------------------|---------------------------------|-----------------|----|----|--------|------|---------------------------------------------|-----------------------------|-------------------------------|---------------------|------|-----------------|
| Internal medicine | 40,683 | 12,463 | 120 (62/58) | 636 | 85 | 1,329 | 184 | 12 | 5,248 | 189 (89/100) | 8,219 (5,531/2,688) | 5,053 | 11 | 74,232 | 2,471,926 |
| General surgery | 8,066 | 9,614 | 2,893 | 335 | 1,870 | 178 | 31 | 0 | 7,495 | 49 (18/31) | 722 | 221 | 0 | 19,382 | 645,421 |
| Obstetrics and gynecology | 5,042 | 6,090 | 263 | 27 | 236 | 0 | 1 | 754 | 6,413 (1,000/5,323) | 351 | 198/153 | 205 | 0 | 0 | 19,382 | 645,421 |
| Urology | 1,586 | 1,245 | 337 | 112 | 148 | 8 | 4 | 0 | 1,176 | 108 (26/82) | 5,066 | 171 | 0 | 0 | 9,951 | 331,368 |
| Otolaryngology | 721 | 2,283 | 333 | 1 | 299 | 0 | 4 | 0 | 668 | 1 | 709 | 0 | 0 | 5,098 | 169,763 |
| Dermatology | 2,156 | 1,270 | 21 | 2 | 28 | 0 | 2 | 436 | 3 | 20 | 21 | 0 | 0 | 4,162 | 138,595 |
| Orthopedic surgery | 750 | 2,672 | 44 | 1 | 77 | 5 | 1 | 193 | 24 | 261 | 63 | 0 | 4,091 | 136,230 |
| Neurosurgery | 330 | 1,062 | 75 | 0 | 183 | 0 | 16 | 0 | 645 | 9 | 207 | 27 | 0 | 2,554 | 85,048 |
| Thoracic surgery | 161 | 589 | 251 | 14 | 277 | 0 | 1 | 502 | 1 | 324 | 35 | 0 | 2,155 | 71,762 |
| Family medicine | 905 | 307 | 0 | 0 | 0 | 0 | 0 | 0 | 23 | 16 | 106 | 110 | 0 | 1,467 | 48,851 |
| Radiology | 541 | 49 | 0 | 0 | 0 | 0 | 0 | 0 | 207 | 5 | 17 | 106 | 0 | 1,125 | 37,463 |
| Plastic surgery | 50 | 680 | 46 | 9 | 84 | 0 | 0 | 117 | 0 | 3 | 3 | 0 | 992 | 33,034 |
| Pediatrics | 227 | 56 | 0 | 0 | 2 | 132 | 24 | 5 | 193 | 0 | 245 | 4 | 0 | 888 | 29,570 |
| Neurology | 171 | 58 | 1 | 0 | 5 | 11 | 7 | 18 | 40 | 1 | 337 | 23 | 0 | 682 | 22,711 |
| Dental department | 123 | 333 | 8 | 0 | 15 | 17 | 0 | 0 | 27 | 0 | 17 | 2 | 0 | 542 | 18,049 |
| Emergency medicine | 74 | 42 | 1 | 0 | 1 | 13 | 3 | 0 | 20 | 9 | 203 | 5 | 0 | 371 | 12,354 |
| Ophthalmology | 140 | 123 | 5 | 0 | 10 | 0 | 0 | 51 | 0 | 10 | 8 | 0 | 347 | 11,555 |
| Rehabilitation medicine | 51 | 17 | 0 | 0 | 1 | 12 | 2 | 3 | 5 | 7 | 46 | 2 | 0 | 146 | 4,862 |
| Laboratory medicine | 5 | - | 0 | 0 | 0 | 0 | 0 | 0 | 71 | 1 | 0 | 0 | 0 | 77 | 2,564 |
| Psychiatry | 43 | 13 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 15 | 2 | 0 | 77 | 2,564 |
| Pathology | 2 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 52 | 1,732 |
| General (NOS) | 12 | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 10 | 0 | 45 | 1,499 |
| Radiation oncology | 16 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 6 | 0 | 43 | 1,432 |
| Preventive medicine | 4 | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 29 | 966 |
| Nuclear medicine | - | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 26 | 0 | 0 | 26 | 866 |
| Anesthesiology | 6 | 8 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 18 | 599 |
| Occupational and environmental medicine | 9 | - | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 11 | 366 |
| Tuberculosis | 1 | - | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 33 |
| Total | 61,875 | 39,015 | 4,399 | 1,137 | 3,322 | 1,908 | 278 | 40 | 17,892 | 6,870 | 16,234 | 10,369 | 33 | 163,372 | 5,440,288 |

LN, with lymph node dissection; non-LN, without lymph node dissection; IF, immunofluorescent; EM, electron microscopy; IHC, immunohisto(cyt)ochemistry; FISH, fluorescence in situ hybridization; SISH, silver in situ hybridization; NOS, not otherwise specified.
RESULTS

Pathologic examination statistics in Korea

In the year 2013, 163,372 pathologic examinations were performed in 103,528 patients (45,897 men and 57,631 women). The mean and median ages of the patients were 51.8 and 53 years, respectively. The skewness and kurtosis of patient age were –0.251 and 2.775, respectively. The pathologic examination data and medical institutions according to administrative district are summarized in Table 2. The total numbers of medical institutions according to administrative district are summarized in Appendix 1. In total, 43.5% of the tertiary hospitals (10/23), 15.6% of the general hospitals (48/307), and 23.8% of the other institutions (12,090/50,868) were located in Seoul. In addition, 32.9% of the surgical pathologic examinations (53,711/163,372) were requested in medical institutions located in Seoul.

Almost all medical and dental departments requested pathologic examinations (Table 3). Internal medicine (74,232, 45.4%), general surgery (34,808, 21.3%), obstetrics and gynecology (19,382, 11.9%), and urology (9,951, 6.1%) were the most common medical departments (84.7%) requesting pathology examinations. A small subset (38/17,892, 0.2%) of immunohistochemical (IHC) stains were not interpreted by qualified doctors (not claimed as “C5575006”) (data not shown). Claims for acetylcholinesterase (C5564) and chloroacetate esterase (C5565) examinations were not found in our data.

Among pathologic examination-associated claims, 162,002 examinations (99.2%) had proper claim codes (examination codes 09 and sub-code 01 (performed in their own institutions) or 02 (performed in outside institutions). Among pathologic examinations, 65.8% were performed in tertiary or general hospitals (39,385 and 67,225 cases, respectively) (Table 4). Almost all examinations claimed by tertiary hospitals were performed in their institute (39,349/39,385, 99.9%), and 85% of examinations claimed by general hospitals were performed (57,163/67,225) in their institute. The other medical institutions claimed considerable pathologic examinations performed by outside services (52,214/55,392, 94.3%).

According to the claims, 76,016 pathologic examinations (44.7%) were performed due to malignancy (disease code “C”) or non-malignant tumorous conditions (disease code “D00-D48”) (Table 5). IHC stains were more frequently performed in tumorous conditions (15,336/17,892, 85.7%). Pathologic examination of biopsy specimens was performed more frequently in non-tumorous conditions (42,661/61,875, 68.9%).

ESD-related statistics

In total, 509 ESDs were performed in 490 patients (341 males

| Table 4. Pathologic examination performing places according to medical institutions |
| Institution | Tertiary hospital | General hospital | Other medical institutions | Total |
|-------------|------------------|------------------|---------------------------|-------|
| In own hospital | 39,349 (99.9) | 57,163 (85.0) | 3,178 (5.7) | 99,690 (61.5) |
| Outside services | 36 (0.1) | 10,062 (15.0) | 52,214 (94.3) | 62,312 (38.5) |
| Total | 39,385 (100) | 67,225 (100) | 55,392 (100) | 162,002 (100) |

Values are presented as number (%).

| Table 5. Disease codes of patients at time of pathologic examinations requests |
| Tumorous condition | Non-tumorous condition | Total |
|-------------------|------------------------|-------|
| Biopsy | 19,214 | 42,661 | 61,875 |
| Resection, non-malignant | 14,900 | 24,115 | 39,015 |
| Resection, malignant (LN/non-LN) | 4,333 (3,203/1,130) | 66 (30/36) | 4,399 (3,233/1,166) |
| Histologic mapping (LN/non-LN) | 1,045 (377/668) | 92 (0/92) | 1,137 (377/760) |
| Frozen sections | 3,041 | 281 | 3,322 |
| IF | 213 | 1,695 | 1,908 |
| EM | 51 | 227 | 278 |
| Enzyme | 0 | 40 | 40 |
| IHC | 15,336 | 2,556 | 17,892 |
| Uterine cervical examinations (smear/liquid) | 3,199 (554/2,645) | 3,671 (708/2,963) | 6,870 (1,259/5,611) |
| Body fluid (conventional/liquid) | 7,137 (4,118/3,019) | 9,007 (6,509/2,588) | 16,234 (10,627/5,607) |
| Aspiration (conventional/liquid) | 4,514 (4,234/280) | 5,855 (6,528/271) | 10,369 (9,782/5,497) |
| HER2 (FISH/SISH) | 33 (9/24) | 0 (0/0) | 33 (9/24) |
| Total | 73,016 | 90,356 | 163,372 |

LN, with lymph node dissection; non-LN, without lymph node dissection; IF, immunofluorescent; EM, electron microscopy; IHC, immunohisto(cyto)chemistry; FISH, fluorescence in situ hybridization; SISH, silver in situ hybridization.
and 149 females) in 109 medical institutions. The median age of the patients was 66 years (age, 29 to 89 years; 1st quantile, 58 years; 3rd quantile, 71.75 years). The majority of patients (n=472) underwent one ESD; 17 patients underwent two ESDs (10 patients underwent simultaneous ESD, while seven patients underwent ESDs at different times), and one patient underwent three ESDs (two ESDs at the same time).

The disease codes noted when patients underwent ESD were as follows: C16 (malignant neoplasm of stomach), 213 patients (43.5%); non-C16, 277 patients (56.5%); D00.2 (carcinoma in situ of stomach), 17 patients; D13.1 (benign neoplasm of stomach), 226 patients; D13.9 (benign neoplasm of ill-defined site within the digestive system), one patient; and other, 33 patients. Disease codes changed after the ESD in 10.5% of the non-C16 patients (29/277): D00.2 to C16, seven patients; D13.1 to D00.2, three patients; D13.1 to C16, 18 patients; and D13.9 to C16, one patient (Table 6).

Twenty-one patients (21/490, 4.3%) underwent gastrectomy following ESD, and all of these patients underwent ESD for only once. Fifteen patients received surgery at the same medical institution where ESD was performed, and six patients received surgery at different medical institutions. The mean time between ESD and surgery was 44 days. Two patients changed diagnosis (benign to malignant) after ESD. One patient underwent ESD and surgery during the same hospitalization period.

For further analysis, 472 patients who underwent ESD for only once were selected (Table 7) (C16, 202 patients; D00.2, 17 patients; D13.1, 220 patients; D13.9, one patient; other, 32 patients). In total, 70.0% of these pathologic examinations (329/472) were requested for histologic mapping (C5508). IHC studies were performed in 22.5% of ESDs (106/472). Approximately one-third of the gastric cancer specimens (66/202, 32.7%) and 15.0% of the gastric benign neoplasm specimens (33/220) were subject to IHC studies.

**DISCUSSION**

In this study, we examined nation-wide statistics regarding surgical pathologic examination. Considering sampling weight, we estimate that 5,440,288 (163,372 × 33.3) pathologic examinations were performed in Korea in 2013. We also surveyed pathologic examinations according to administrative district, requesting department, type of medical institutions, and patient conditions. These data will be useful for future planning and allocation of resources in the field of pathology and for the Korean Society of Pathologists.

There have been several reports regarding diagnostic discrepancies between endoscopic forceps biopsy and ESD, as well as between ESD and surgery. Recently, two large, single-center, retrospective studies revealed that 22.8% (465/2,041) and 31.7% (69/220) of the gastric cancer cases were malignant.

**Table 6.** Changes of disease codes after endoscopic submucosal dissections

| Disease code at ESD | Disease code after ESD | Unchanged | Changed to C16 | Changed to D00.2 |
|---------------------|------------------------|-----------|----------------|-----------------|
| D00.2               |                        | 10        | 7              |                 |
| D13.1               |                        | 205       | 18             | 3               |
| D13.9               |                        | 0         | 1              | 0               |
| Others              |                        | 33        | 0              | 0               |
| Total               |                        | 248       | 26             | 3               |

ESD, endoscopic submucosal dissections.

**Table 7.** Pathologic examination codes and number of immunohistochemical stains according to diseases codes at endoscopic submucosal dissections

| Pathologic examination codes | C16 | D00.2 | D13.1 | D13.9 | Others | Total |
|-----------------------------|-----|-------|-------|-------|--------|-------|
| C5500                       | 6   | 0     | 5     | 0     | 0      | 11    |
| C5508                       | 158 | 3     | 139   | 1     | 28     | 329   |
| C5916                       | 16  | 3     | 51    | 0     | 2      | 72    |
| C5917                       | 22  | 11    | 25    | 0     | 2      | 60    |
| Total                       | 202 | 17    | 220   | 1     | 32     | 472   |

No. of immunohistochemical stains

|                      | 0    | 1     | 2     | 3     | 4     | 5     | Total |
|----------------------|------|-------|-------|-------|-------|-------|-------|
|                      | 136  | 2     | 12    | 2     | 3     | 5     | 366   |
|                      | 42   | 1     | 2     | 0     | 2     | 3     | 69    |
|                      | 12   | 0     | 1     | 3     | 0     | 0     | 19    |
|                      | 2    | 0     | 1     | 0     | 0     | 0     | 3     |
|                      | 3    | 0     | 0     | 0     | 0     | 0     | 3     |
|                      | 5    | 0     | 0     | 0     | 0     | 0     | 5     |
| Total                | 202  | 17    | 220   | 1     | 32    | 472   |
Surgical Pathology Status in HIRA Data

• 209

(587/1,850) of cases changed diagnosis after endoscopic resection in Asan Medical Center (Seoul, Korea) and Samsung Medical Center (Seoul, Korea), respectively.6,7 Our HIRA-NPS data revealed that only 10.9% of nation-wide cases experienced a change of diagnosis after ESD. HIRA-NPS data does not include detailed pathologic diagnosis information such as tumor size, tumor differentiations, dysplastic degrees, etc., so further analysis for clarifying such differences was limited. Shin et al.7 reported that complete resection rates were not different according to absolute or expanded ESD indications, though their data were not HIRA data. Although HIRA-NPS data was not available for a sufficient number of ESD patients (509 ESDs in 490 patients) to allow for an accurate assessment, and only limited clinicopathological information was available, the problems associated with the relatively few number of patients can be overcome through further research using the raw HIRA data.

Histologic mapping of ESD specimens is recommended by The Gastrointestinal Pathology Study Group of the Korean Society of Pathologists.8 However, 30.2% (143/472) of ESD specimens were not claimed as “C5508” (histologic mapping without lymph node dissection). Because precise histologic diagnosis of the ESD specimen is essential to treat patients, we suggest that nationwide surveys be conducted in order to assure quality of pathologic examination of ESD specimens. IHC staining was performed in 22.5% (106/472) of ESD cases. Unlike tissue immunofluorescent microscopic examinations or enzyme histochemistries, HIRA data does not list the specific antibodies used for IHC stains. Thus, further analysis of IHC studies using HIRA data was limited.

The main limitations of our analysis are statistical issues based on probability sampling. Our estimates were calculated from statistically extracted data from one year. Further analysis using HIRA raw data will be needed to decrease the statistical errors and bias and to evaluate changes over time. During our analysis, we experienced many challenges; therefore, we provide advice and guidance for other researchers who would like to analyze the HIRA database.

(1) It is essential to understand the framework of the HIRA database.9 The HIRA data is intended for insurance claims and not for research. Detailed clinicopathological data are not provided. (2) Sample data are not appropriate for analyses requiring long-term follow-up. (3) Some claim codes have sub-codes (in most cases, additional charges by experts). (4) Disease codes and claims are not always accurate. (5) The analysis system should have at least 32 GB of main memory. (6) It is helpful to create relatively small data tables to decrease operation time. In the usual setting, R should use only one CPU core during calculation.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Appendix 1. Numbers of medical institutions according to administrative districts

| Administrative district | Tertiary hospital | General hospital | Other institutions (clinic) |
|-------------------------|------------------|-----------------|-----------------------------|
| Seoul                   | 10               | 48              | 12,090 (6,952)              |
| Busan                   | 3                | 24              | 3,567 (2,048)               |
| Incheon                 | 2                | 15              | 2,376 (1,388)               |
| Daegu                   | 1                | 11              | 2,542 (1,538)               |
| Gwangju                 | 1                | 22              | 1,584 (962)                 |
| Daejeon                 | 1                | 8               | 1,590 (981)                 |
| Ulsan                   | 0                | 4               | 1,012 (546)                 |
| Gyeonggi-do             | 1                | 55              | 10,424 (5,883)              |
| Gangwon-do              | 1                | 14              | 1,377 (707)                 |
| Chungcheongbuk-do       | 1                | 10              | 1,488 (791)                 |
| Chungcheongnam-do       | 0                | 12              | 2,019 (1,013)               |
| Jeollabuk-do            | 1                | 11              | 2,197 (1,110)               |
| Jeollanam-do            | 0                | 22              | 2,048 (913)                 |
| Gyeongsangbuk-do        | 0                | 19              | 2,558 (1,206)               |
| Gyeongsangnam-do        | 1                | 25              | 2,977 (1,521)               |
| Jeju                    | 0                | 7               | 575 (330)                   |
| Sejong-si               | 0                | 0               | 105 (57)                    |
| Total                   | 23               | 307             | 50,868 (27,846)             |
Aberrant Blood Vessel Formation Connecting the Glomerular Capillary Tuft and the Interstitium Is a Characteristic Feature of Focal Segmental Glomerulosclerosis-like IgA Nephropathy

Beom Jin Lim1,2 · Min Ju Kim1 · Soon Won Hong1 · Hyeon Joo Jeong1

1Department of Pathology, 2Severance Institute for Vascular and Metabolic Research, Yonsei University College of Medicine, Seoul; 3Department of Pathology, Gachon University Gil Medical Center, Incheon, Korea

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Corresponding Author
Hyeon Joo Jeong, MD
Department of Pathology, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea
Tel: +82-2-2228-1766
Fax: +82-2-362-0860
E-mail: jeong10@yuhs.ac

IgA nephropathy (IgAN) is characterized by dominant or co-dominant IgA deposits in the mesangial matrix and is usually accompanied by focal or diffuse mesangial proliferation. However, IgAN has a variety of histologic features ranging from normal to minimal glomerular alterations to diffuse endocapillary and crescentic glomerulonephritis.

Glomerular segmental sclerosis is not unusual in biopsy samples that are diagnosed as IgAN, but it is usually associated with severe mesangial proliferation and/or advanced tubulointerstitial fibrosis. Haas1–4 reported a form of segmental sclerosis in IgAN that did not accompany mesangial proliferation and demonstrated clinical features that were indistinguishable from idiopathic focal segmental glomerulosclerosis (FSGS). Of the 18 cases presented in his study, 82% had nephrotic syndrome and all but one case had favorable prognoses. Another description of segmental sclerosis without significant mesangial proliferation was reported by Weber et al.2 In their series of 26 cases, nephrotic syndrome was present in 24%, diffuse effacement of foot processes in only one case and the clinical outcomes were worse than in cases without FSGS. These two contrasting studies suggest that cases of mild IgAN with FSGS that are morphologically similar but have different pathogeneses may have different clinical outcomes. We recently recognized aberrant blood vessels running through the adhesion sites of sclerosed tufts and Bowman’s capsule in IgAN cases with mild glomerular histologic change. Methods: To characterize aberrant blood vessels in relation to segmental sclerosis, we retrospectively reviewed the clinical and histologic features of 51 cases of FSGS-like IgAN and compared them with 51 age and gender-matched idiopathic FSGS cases. Results: In FSGS-like IgAN, aberrant blood vessel formation was observed in 15.7% of cases, 1.0% of the total glomeruli, and 7.3% of the segmentally sclerosed glomeruli, significantly more frequently than in the idiopathic FSGS cases (p = .009). Aberrant blood vessels occasionally accompanied mild cellular proliferation surrounding penetrating neovessels. Clinically, all FSGS-like IgAN cases had hematuria; however, nephrotic range proteinuria was significantly less frequent than idiopathic FSGS. Conclusions: Aberrant blood vessels in IgAN are related to glomerular capillary injury and may indicate abnormal repair processes in IgAN.

Key Words: Glomerulosclerosis, focal segmental; Glomerulonephritis, IgA; Kidney glomerulus; Neovascularization
MATERIALS AND METHODS

Materials

A total of 565 cases with diagnoses of IgAN in native kidneys were retrieved from the renal biopsy registry of the Department of Pathology, Yonsei University Health System, between 1999 and 2008. Among these, 92 cases were diagnosed as IgAN, subclass II, according to the Haas classification. We selected 51 IgAN cases with focal and segmental sclerosis. The exclusion criteria included (1) significant glomerular mesangial proliferation of more than three cells in one mesangial area distant from the vascular hilum in 2-3-μm-thick periodic acid–Schiff (PAS)-stained sections; (2) endocapillary proliferation; (3) global glomerulosclerosis or tubular atrophy of 40% or more; or (4) insufficient sample size of less than six glomeruli on light microscopy. For comparison, age- and gender-matched idiopathic FSGS cases from the same period were collected from our renal biopsy registry. The same exclusion criteria were applied to these cases.

Tissue processing of renal biopsies

All biopsy samples were examined using light, immunofluorescent, and electron microscopy. For light microscopic examination, 2–3-μm-thick sections were stained with hematoxylin and eosin, PAS, aldehyde fuchsin orange G, and methenamine silver methods. Two to four tissue sections were mounted per glass slide, and two slides were prepared for each stain, resulting in eight slides for each biopsy. For immunofluorescence examination, snap-frozen 3-μm-thick sections were mounted in optimal cold temperature compound and stained with antibodies against IgG, IgA, IgM, C3, C4, C1q, and fibrinogen (Dako Cytomation, Glostrup, Denmark). For electron microscopy, fresh renal tissue was cut at the time of biopsy, and one to three blocks of 1 mm³ renal tissue were double fixed with glutaraldehyde and osmium tetroxide, routinely processed, and stained with uranyl acetate and lead citrate.

Assessment of renal histology

An average of 26.7 consecutive sections (range, 18 to 32) were examined in each case. All glomeruli in each section were traced. The total glomeruli and glomeruli showing segmental sclerosis were counted. The segmental sclerosis was subtyped in each case as classic, perihilar, tip, cellular or collapsing variants and were numbered. In segmental sclerosis, capillary adhesion to Bowman’s capsule was observed in 95 glomeruli (86.4% of the segmentally sclerosed glomeruli) in 49 cases.

RESULTS

Morphologic features of segmental sclerosis in FSGS-like IgAN

We observed an average of 16.3 glomeruli in each case of FSGS-like IgAN. Of the 829 total glomeruli, 110 (13.3%) showed segmental sclerosis. The most common subtype of segmental sclerosis was classic type, followed by tip and perihilar types. Hyalinosis was present in one glomerulus. Glomerular capillary tuft adhesion to Bowman’s capsule was observed in 95 glomeruli (86.4% of the segmentally sclerosed glomeruli) in 49 cases. Among them, nine cases (8.2% of the total segmentally sclerosed glomeruli) demonstrated capillary-sized blood vessels connecting the segmentally sclerosed tuft and periglomerular interstitium. These vessels began in the sclerosed tufts, ran perpendicularly through the gap of Bowman’s capsule, and merged...
into the capillary networks of the interstitium. The vessels were surrounded by normal-looking glomerular capillary loops in five glomeruli, but accompanied endocapillary proliferations in four glomeruli (Fig. 1).

Comparison of segmental sclerosis between FSGS-like IgAN and idiopathic FSGS

In age and gender-matched idiopathic FSGS cases, segmental sclerosis was observed in 119 out of 746 glomeruli (16.0%). When segmental sclerosis of IgAN was compared with that of idiopathic FSGS, the mean percentages of glomeruli with tuft adhesion, aberrant vessel formation, and glomerular crescents were significantly higher in the FSGS-like IgAN group. Aberrant blood vessels found in FSGS were similar to those seen in IgAN in morphology but were identified in only one glomerulus (0.8% of the segmentally sclerosed glomeruli). The mean percentages of glomerular hyalinosis, the degrees of interstitial fibrosis, tubular atrophy, interstitial inflammation, and arteriolar hyalinosis were not significantly different between the two groups (Table 1).

Clinically, hematuria was present in 100% of the FSGS-like IgAN patients, whereas 51.0% of the idiopathic FSGS patients had hematuria. Proteinuria was more prevalent and severe in idiopathic FSGS: 91.1% of patients had proteinuria and 48.9% were in the nephrotic range, whereas proteinuria was present in

Fig. 1. Consecutive photos of two glomeruli showing extra-efferent vessel formation in focal segmental glomerulosclerosis-like IgA nephropathy. (A, B) Extra vessels connecting the segmentally sclerosed portions of glomerular tufts and the extraglomerular space through the gap of Bowman’s capsule were observed when tracing the serial sections. These vessels were not connected to afferent or efferent arterioles in the vascular pole (solid arrows, extra vessels; open arrows, vascular poles). (B) Surrounding glomerular tufts showed capillary and endothelial proliferations in some cases (arrowhead) (periodic acid-Schiff, aldehyde fuchs in orange G, and methenamine silver stain).
79.9% of FSGS-like IgAN patients with nephrotic range in 2.0%. Other clinical parameters including mean serum creatinine levels were similar in both groups (Table 2).

### DISCUSSION

Aberrant vessels were more frequently observed in cases of FSGS-like IgAN than in idiopathic FSGS. Otherwise, the morphology of segmental sclerotic glomeruli was indistinguishable in two diseases, and the most common form was the classic type. Considering that more than 25 sections were examined, these features were rare and may be under-recognized or unnoticed in routine practice. Clinically, our cases were different from those reviewed by Haas et al. but similar to those reviewed by Weber et al. The frequency of hematuria was higher and that of nephrotic range proteinuria was lower than observed by Haas. Associations of nephrotic range proteinuria have been reported in IgAN with normal histology. However, it has not been resolved whether this entity is a subtype of IgAN or an overlap of IgAN and minimal change disease. Likewise, it is possible that the cases of FSGS-like IgAN described by Haas et al. were subtypes of IgAN or represented the overlap of IgAN and minimal change disease/FSGS. By contrast, consistent hematuria may suggest the involvement of capillary loop injury in FSGS-like IgAN.

Extra new vessel formation is rarely reported in animal models or in human diabetic nephropathy. Kriz et al. proposed that “extraterritorial glomerular capillaries” that connect the glomerular space and periglomerular interstitium are responsible for the development of interstitial fibrosis in a hypertensive rat FSGS model. The extra vessels were located in the glomerular hilum in human diabetic nephropathy and did not cause rupture of the capillary loops, and most were connected to the second- and third-order branches of the afferent arteriole and drained into the peritubular capillaries. The number of extra vessels ranged from

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**Table 1. Histomorphologic characteristics of FSGS-like IgAN and idiopathic FSGS**

| Variable                        | FSGS-like IgAN | Idiopathic FSGS | p-value |
|---------------------------------|----------------|-----------------|---------|
| No. of total glomeruli (per case) | 829 (16.3)     | 746 (14.6)      | -       |
| Glomeruli with segmental sclerosis, n (%) | 110 (13.3)     | 119 (16.0)      | -       |
| Classic/Perihilar/Tip/Collapsible/Collapsing type | 56/14/42/0/0 | 73/7/33/0/6    | -       |
| Segmental sclerosis in each case (%) | 14.6 ± 7.3   | 19.5 ± 16.4    | .451    |
| Glomerular hyalinosis in each case (%) | 0.0 ± 0.1      | 0.8 ± 3.3       | .177    |
| Capillary loop adhesion in each case (%) | 13.2 ± 7.8  | 6.5 ± 8.7       | <.001   |
| Neovessel formation in each case (%) | 1.2 ± 3.1    | 0.2 ± 1.1       | .009    |
| Glomerular crescents               |                |                 | <.001   |
| Absent                            | 39             | 51              |         |
| Present                           | 12             | 0               |         |
| Interstitial fibrosis             |                |                 | >.999   |
| Absent or mild                    | 49             | 49              |         |
| Moderate to severe                | 2              | 2               |         |
| Tubular atrophy                   |                |                 | .678    |
| Absent or mild                    | 49             | 47              |         |
| Moderate to severe                | 2              | 4               |         |
| Interstitial inflammation         |                |                 | >.999   |
| Absent or mild                    | 47             | 47              |         |
| Moderate to severe                | 4              | 4               |         |
| Arteriolar hyalinosis             |                |                 | .463    |
| Absent                            | 39             | 42              |         |
| Present                           | 12             | 9               |         |

FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy. *Mann-Whitney U test; †Pearson's chi-square test with Fisher exact test.

**Table 2. Clinical characteristics of FSGS-like IgAN and idiopathic FSGS**

| Variable                        | FSGS-like IgAN | Idiopathic FSGS | p-value |
|---------------------------------|----------------|-----------------|---------|
| No. of cases                    | 51             | 51              |         |
| Male:Female                     | 25:26          | 24:27           |         |
| Age, mean (range, yr)           | 35.1 (7–65)    | 35.6 (7–67)     |         |
| Proteinuria*                    | 39 (79.9)      | 41 (91.1)       | 0.124   |
| Nephrotic range                 | 1 (2.0)        | 22 (48.9)       | 0.000   |
| Hematuria*                      | 51 (100)       | 26 (51.0)       | 0.000   |
| Serum creatinine level (mg/dL)  | 1.0 ± 0.2      | 1.2 ± 0.7       | 0.090   |

Values are presented as number (%) or mean ± standard deviation unless otherwise indicated.

FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy. *Number of cases; †Pearson's chi-square test with Fisher exact test; ‡Mann-Whitney U test.
several to more than 10 per glomerulus. Rare extra vessels have been observed in a variety of cases, also localized within the glomerular hilum.\(^{10,11}\) However, the aberrant vessels observed in our study differed from those in diabetic nephropathy in location and numbers. Extra vessels similar to our cases can be seen in glomerulonephritis characterizing small vessel vasculitis such as Henoch-Schönlein purpura nephritis, antineutrophil cytoplasmic antibody–associated nephritis, and lupus nephritis. In these cases, new vessel formation was observed especially inside or near crescents.

Glomerular capillary loop sclerosis and adhesion were grouped together in a proposed Oxford classification of IgAN,\(^{12}\) mainly to increase reproducibility between observers. However, not only are they morphologically distinct but they also differ in pathomechanisms. Glomerular capillary loop sclerosis is characterized by capillary loop collapse and accumulation of scleroprotein inside the glomerular capillary basement membrane. It can be caused by ischemia, toxins, or drugs, but the glomerular basement membrane integrity is essentially preserved. In contrast, glomerular capillary adhesion to Bowman’s capsule produces a break in glomerular basement membrane integrity and may develop with or without glomerular capillary rupture. Therefore, adhesions and new vessels in IgAN might be signs of glomerular capillary injury and rupture. The presence of endocapillary proliferation and small glomerular crescents surrounding vessels supports this possibility. Glomerular capillary loop injury may be triggered by immune deposits along the capillary loops; however, our cases had localized mesangial deposits without peripheral capillary IgA deposits. Glomerular capillary basement membranes may be fragile and likely to break without deposits, since glomerular basement membrane thinning or attenuation is frequent in IgAN.\(^{13}\) Mesangial cells have been shown to significantly down regulate vascular endothelial growth factor A mRNA and increase inducible nitric oxide synthase mRNA when incubated with aberrantly glycosylated IgA. Therefore, these molecules may be involved in the evolution of abnormal repair in IgAN.\(^{14}\) It is unclear how aberrant vessels affect glomerular filtration and nearby structures, but they may contribute to interstitial inflammation and fibrosis by directly delivering pathogenic filtrate to the peritubular capillaries and interstitium.\(^{8}\)

In conclusion, aberrant blood vessels were observed in segmentally sclerosed glomerular tufts in FSGS-like IgAN. Such structures indicate aberrant repair processes in glomerular capillaries. Further clinical and experimental studies may be needed to elucidate this faulty repair mechanism.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Core Needle Biopsy Is a More Conclusive Follow-up Method Than Repeat Fine Needle Aspiration for Thyroid Nodules with Initially Inconclusive Results: A Systematic Review and Meta-Analysis

Jung-Soo Pyo · Jin Hee Sohn
Guhyun Kang

Department of Pathology, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul; Department of Pathology, Inje University Sanggye Paik Hospital, Seoul, Korea

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Corresponding Author
Jin Hee Sohn, MD
Department of Pathology, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, 29 Saemunan-ro, Jongno-gu, Seoul 03181, Korea
Tel: +82-2-2001-2391
Fax: +82-2-2001-2398
E-mail: jhpath.sohn@samsung.com

Background: This study investigated the appropriate management of thyroid nodules with prior non-diagnostic or atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) through a systematic review and meta-analysis. Methods: This study included 4,235 thyroid nodules from 26 eligible studies. We investigated the conclusive rate of follow-up core needle biopsy (CNB) or repeat fine needle aspiration (rFNA) after initial fine needle aspiration (FNA) with non-diagnostic or AUS/FLUS results. A diagnostic test accuracy (DTA) review was performed to determine the diagnostic role of the follow-up CNB and to calculate the area under the curve (AUC) on the summary receiver operating characteristic (SROC) curve. Results: The conclusive rates of follow-up CNB and rFNA after initial FNA were 0.879 (95% confidence interval [CI], 0.801 to 0.929) and 0.684 (95% CI, 0.627 to 0.736), respectively. In comparison of the odds ratios of CNB and rFNA, CNB had more frequent conclusive results than rFNA (odds ratio, 5.707; 95% CI, 2.530 to 12.875). Upon subgroup analysis, follow-up CNB showed a higher conclusive rate than rFNA in both initial non-diagnostic and AUS/FLUS subgroups. In DTA review of follow-up CNB, the pooled sensitivity and specificity were 0.94 (95% CI, 0.88 to 0.97) and 0.88 (95% CI, 0.84 to 0.91), respectively. The AUC for the SROC curve was 0.981, nearing 1. Conclusions: Our results show that CNB has a higher conclusive rate than rFNA when the initial FNA produced inconclusive results. Further prospective studies with more detailed criteria are necessary before follow-up CNB can be applied in daily practice.

Key Words: Thyroid nodule; Non-diagnostic or atypia of undetermined significance/follicular lesion of undetermined significance; Follow-up core needle biopsy; Repeat fine-needle aspiration; Meta-analysis

Papillary thyroid carcinoma, which has recently increased in incidence, is the most common malignant tumor in endocrine system.1 The cause for the increased incidence of papillary thyroid carcinoma is not fully understood.1 One possible cause is the improvement in ultrasonography and computed tomography.2,3 In daily practice, the treatment and follow-up for thyroid nodules are based on the results of initial fine needle aspiration (FNA). According to current guidelines,4-6 FNA is recommended as the initially performed modality, and additional testing is suggested as indicated by the initial FNA results.

In daily practice, repeat FNA (rFNA) is recommended for thyroid nodules of non-diagnostic or atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS). In addition, for definite diagnosis of thyroid nodules, BRAFV600E mutation test or diagnostic surgery can also be performed. In previous studies, the rates of non-diagnostic and AUS/FLUS were 5%–17% and 3%–18%, respectively.7-9 For thyroid nodule with non-diagnostic result, the possibility of an inconclusive reading might also be higher with rFNA. Although rFNA is recommended for non-diagnostic or AUS/FLUS thyroid nodules in the current guidelines, other modalities, such as core needle biopsy (CNB) or combination of FNA and CNB, have been introduced in recent reports.10,11 However, the effectiveness or diagnostic role of these follow-up modalities has not been fully elucidated.

In this study, follow-up CNB was defined as CNB performed after initial non-diagnostic or AUS/FLUS findings. We investigated the conclusive rates of the follow-up procedures of CNB and rFNA in thyroid nodule with initial non-diagnostic or AUS/FLUS finding through a systematic review and meta-analysis. Indeed, the diagnostic test accuracy (DTA) review was performed to determine the diagnostic accuracy of follow-up CNB in thy-
roid nodules with initial inconclusive results.

**MATERIALS AND METHODS**

**Literature search and selection criteria**

Relevant articles were obtained from a search of PubMed and MEDLINE databases through December 31, 2015. The search was performed using ‘thyroid,’ ‘core needle biopsy,’ and ‘fine needle aspiration’ as search terms. The titles and abstracts of all returned articles were screened for exclusion. To find additional eligible studies, review articles were also screened. Search results were then reviewed and included if (1) initial FNA for a thyroid nodule was performed, and (2) there was information about CNB or FNA as a follow-up study for thyroid nodules with initial non-diagnostic or AUS/FLUS results. Articles were excluded if they were (1) non-original articles or case reports or (2) non-English language publications.

**Data extraction**

The following information was collected from the full texts of eligible studies and verified: name of first author, publication year, study location, number of patients analyzed, and method and results of initial and follow-up studies. We did not define a minimal number of patients to be included in a study. Any disagreements were resolved by consensus.

**Statistical analysis**

Data were analyzed using the Comprehensive Meta-Analysis software package (Biostat, Englewood, NJ, USA). We evaluated the conclusive rates of follow-up studies with follow-up CNB or rFNA after initial FNA with non-diagnostic or AUS/FLUS results. The conclusive results included benign, follicular neoplasm or suspicious for follicular neoplasm, suspicious for malignancy, and malignancy categories. The conclusive rates were measured by dividing the number of conclusive results into the total number of cases with a follow-up study. The heterogeneity between eligible studies was assessed using Q and I² statistics and presented using p-values. A sensitivity analysis was performed to assess the impact of each study on the combined effect and the heterogeneity of eligible studies. To identify any publication bias, Egger’s test and Begg’s funnel plot were initially performed. When a significant publication bias was found, the fail-safe N and trim-and-fill tests were additionally conducted to confirm the degree of bias. The results were considered statistically significant at p < 0.05.

A DTA review was conducted using the Meta-Disc program ver. 1.4. The forest plots of pooled sensitivity and specificity and the summary receiver operating characteristic (SROC) curve were determined as described previously.13 The diagnostic odds ratio (OR) and the value of the area under the curve (AUC) on SROC were investigated.

**RESULTS**

**Selection and characteristics of studies**

In the current study, 356 reports were identified in the database search. Among the search results, 209 reports were excluded due to insufficient information. In addition, 121 reports were excluded for the following reasons: focusing on other diseases (n = 86), non-original articles (n = 17), duplicate articles (n = 13), and articles in a language other than English (n = 5). Twenty-six eli-
gible studies and 4,253 thyroid nodules were ultimately included in the current study (Table 1, Fig. 1). The characteristics of the included studies are shown in Table 1.

Table 1. Main characteristics of eligible studies

| Study          | Location | Diagnosis of initial FNA | Follow-up study | No. | Results of follow-up study |
|----------------|----------|--------------------------|-----------------|-----|---------------------------|
| Anderson et al. (2014) | USA      | Non-diagnostic           | FNA             | 336 | 263 73                     |
| Baloch et al. (2003)    | USA      | Non-diagnostic           | FNA             | 123 | 92 31                     |
| Chen et al. (2012)      | USA      | AUS/FLUS                 | FNA             | 26  | 17 9                      |
| Choi et al. (2014)      | Korea    | Non-diagnostic           | CNB             | 128 | 120 8                     |
| Choi et al. (2014)      | Korea    | AUS/FLUS                 | CNB             | 107 | 103 4                      |
| Dincer et al. (2013)    | Turkey   | AUS/FLUS                 | FNA             | 74  | 51 23                     |
| Gocun et al. (2014)     | Turkey   | AUS/FLUS                 | FNA             | 118 | 73 45                     |
| Gwoen et al. (2013)     | Korea    | AUS/FLUS                 | FNA             | 86  | 81 5                      |
| Ho et al. (2014)        | USA      | AUS/FLUS                 | FNA             | 116 | 74 42                     |
| Hyeon et al. (2014)     | Korea    | AUS/FLUS                 | FNA             | 274 | 214 60                    |
| Jo et al. (2011)        | USA      | Non-diagnostic           | FNA             | 363 | 267 96                    |
| Lee et al. (2015)       | Korea    | AUS/FLUS                 | CNB             | 34  | 28 6                      |
| Lee et al. (2014)       | Korea    | Non-diagnostic           | CNB             | 118 | 74 44                     |
| Moon et al. (2015)      | Korea    | AUS/FLUS                 | FNA             | 246 | 185 61                    |
| Moslavac et al. (2012)  | Croatia  | Non-diagnostic           | FNA             | 38  | 31 7                      |
| Na et al. (2015)        | Korea    | AUS/FLUS                 | CNB             | 158 | 110 48                    |
| Na et al. (2012)        | Korea    | Non-diagnostic           | CNB             | 45  | 43 2                      |
| Na et al. (2011)        | Korea    | AUS/FLUS                 | CNB             | 45  | 32 13                     |
| Na et al. (2011)        | Korea    | AUS/FLUS                 | CNB             | 104 | 82 22                     |
| Na et al. (2011)        | Korea    | AUS/FLUS                 | FNA             | 104 | 75 29                     |
| Nagarkatti et al. (2013)| USA      | AUS/FLUS                 | FNA             | 51  | 28 23                     |
| Oertel et al. (2007)    | USA      | Non-diagnostic           | FNA             | 27  | 23 4                      |
| Park et al. (2011)      | Korea    | Non-diagnostic           | CNB             | 54  | 53 1                      |
| Park et al. (2015)      | Korea    | AUS/FLUS                 | FNA             | 236 | 155 81                    |
| Samir et al. (2012)     | USA      | Non-diagnostic           | CNB             | 69  | 51 18                     |
| Sullivan et al. (2014)  | USA      | AUS/FLUS                 | FNA             | 69  | 36 33                     |
| Trimkol et al. (2015)   | Italy    | Indeterminate            | CNB             | 198 | 125 73                    |
| Yoon et al. (2013)      | Korea    | Non-diagnostic           | CNB             | 116 | 108 8                     |
| Yoon et al. (2011)      | Korea    | Non-diagnostic           | CNB             | 99  | 91 8                      |

FNA, fine needle aspiration; CNB, core needle biopsy; AUS/FLUS, atypia/follicular lesion of undetermined significance.

Table 2. Conclusive rates of second CNB and repeat FNA after prior FNA with non-diagnostic or AUS/FLUS significance in thyroid nodules

| No. of subsets | No. of patients | Fixed effect model (95% CI) | Heterogeneity (p-value) | Random effect model (95% CI) | Egger’s test |
|----------------|-----------------|-----------------------------|-------------------------|----------------------------|--------------|
| Overall        | 35              | 4,566                        | 0.690 (0.675–0.704)     | <.001 | 0.748 (0.701–0.791)     | .009         |
| Second CNB     | 11              | 1,134                        | 0.775 (0.745–0.802)     | <.001 | 0.879 (0.801–0.929)     | <.001        |
| Non-diagnostic | 6               | 533                          | 0.897 (0.864–0.923)     | <.001 | 0.927 (0.847–0.966)     | .122         |
| AUS/FLUS       | 5               | 601                          | 0.712 (0.671–0.749)     | <.001 | 0.794 (0.675–0.877)     | .030         |
| Repeat FNA     | 24              | 3,432                        | 0.670 (0.653–0.686)     | <.001 | 0.684 (0.627–0.736)     | .651         |
| Non-diagnostic | 11              | 1,739                        | 0.674 (0.650–0.697)     | <.001 | 0.699 (0.596–0.785)     | .773         |
| AUS/FLUS       | 13              | 1,693                        | 0.666 (0.642–0.689)     | <.001 | 0.673 (0.608–0.732)     | .719         |

CNB, core needle biopsy; FNA, fine needle aspiration; AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; CI, confidence interval.

Higher conclusive rate in follow-up CNB than in rFNA

For initial FNA with non-diagnostic or AUS/FLUS, follow-up studies using CNB or rFNA showed an overall conclusive
rate of 0.690 (95% confidence interval [CI], 0.675 to 0.704) and 0.748 (95% CI, 0.701 to 0.791) with the fixed and random effect models, respectively. For follow-up CNB, the conclusive rate was 0.775 (95% CI, 0.745 to 0.802) and 0.879 (95% CI, 0.801 to 0.929) with the fixed and random effect models, respectively. In rFNA, the conclusive rate was 0.670 (95% CI, 0.653 to 0.686) and 0.684 (95% CI, 0.627 to 0.736) with the fixed and random effect models, respectively. The ranges of conclusive rates were 0.631–0.981 and 0.286–0.942 for follow-up CNB and rFNA, respectively. Follow-up CNB showed a higher conclusive rate compared with rFNA (OR, 5.707; 95% CI, 2.530 to 12.875). The heterogeneity of eligible studies was significant in both follow-up CNB and rFNA groups (I² = 90.4%, p < 0.001 and I² = 90.3%, p < 0.001, respectively). In sensitivity analysis, no study had an effect on the concordance rates for either follow-up CNB (range, 0.864 to 0.896) or rFNA (range, 0.670 to 0.697). Subgroup analysis revealed a significant difference in conclusive rate between follow-up CNB and rFNA in both non-diagnostic (0.927; 95% CI, 0.848 to 0.966 vs 0.699; 95% CI, 0.596 to 0.785) and AUSL/FLUS cases (0.794; 95% CI, 0.675 to 0.877 vs 0.673; 95% CI, 0.608 to 0.732) with the random effect model (Table 2).

Egger’s test revealed that the follow-up CNB group showed a significant publication bias (p = .009). Additionally, fail-safe N and trim-fill tests were performed for confirmation of the degree of publication bias in the CNB group. The number of missing studies that would produce a p-value higher than alpha was 5,401 on the fail-safe N test. Because there were 11 observed studies, the publication bias was not large. In addition, the trim and fill test showed no significant difference between the observed and adjusted values. Therefore, we concluded that the publication bias in the follow-up CNB group was not significant through interpretation of Egger’s test, Beggs funnel plot, the fail-safe N test, and the trim-fill test. In the rFNA group, there was no significant publication bias according to Egger’s test (p = .651) or Beggs funnel plots.

DTA review of follow-up CNB as a follow-up study

For confirmation of the diagnostic accuracy of follow-up CNB, we conducted a DTA review. The pooled sensitivity and specificity values were 0.94 (95% CI, 0.88 to 0.97) and 0.88 (95% CI, 0.84 to 0.91), respectively (Fig. 2). The sensitivity and speci-
The summary receiver operating characteristics (SROC) curve of follow-up core needle biopsy for prediction of papillary thyroid carcinoma after prior fine needle aspiration with non-diagnostic or atypia/follicular lesion of undetermined significance in thyroid nodules. AUC, area under the curve.

The conclusive and inconclusive rates of rFNA and follow-up CNB were compared. The conclusive rate and specificity of eligible studies ranged from 0.70 to 0.97 and from 0.45 to 1.00, respectively. On the SROC curve, the value of AUC was 0.981 (Fig. 3). In addition, the diagnostic OR was 448.73 (95% CI, 36.63 to 5,497.51).

**DISCUSSION**

The current guidelines recommend FNA as the initial test for thyroid nodules.43,45 Although rFNA was applied to thyroid nodules with non-diagnostic and AUS/FLUS findings,4,6 the effectiveness of rFNA has not been fully elucidated. The present study is the first meta-analysis and DTA review of published studies on the diagnostic role of follow-up CNB compared with rFNA after initial FNA with non-diagnostic or AUS/FLUS results.

In the assessment of thyroid nodule, FNA is the most cost-effective initial test. Recently, FNA using ultrasonography has produced a higher quality of specimens for more precise diagnosis.10 However, some cases were non-diagnostic or AUS/FLUS, and the rates of inconclusive results ranged from 10%–33.6% in previous studies.6,8,9,40 Although the current guidelines recommend rFNA as a follow-up study for thyroid nodules with initial inconclusive results,14 rFNA does not ensure conclusive results. The inconclusive rates of rFNA in previous studies ranged from 9.9% to 72.0%.18,29,34,36 In the current meta-analysis, the rate of inconclusive results for rFNA was 31.6%. If the cellularity of initial FNA is low, rFNA can also show low cellularity. In a previous study, rFNA was the most significant risk factor affecting repeat non-diagnostic results.18 This situation might be caused by the nature of the thyroid nodule, including factors such as intratumoral calcification and cystic change.41-43 In addition, the quality of the specimen might also be affected by the experience of the operator. Consequently, diagnostic surgery is recommended for thyroid nodule with repeat inconclusive results, and the incidence of diagnostic surgery has been reported as 22.2%–94.7%.24,44-46 Therefore, to reduce the inconclusive rate, an alternative follow-up study, such as CNB, could be considered for thyroid nodules with inconclusive readings in initial FNA.

Considering the results of a previous meta-analysis, histologic examination using CNB as an initial test might be useful for obtaining conclusive results.47 Other previous comparative studies of FNA and CNB as an initial test have reported that CNB has a suspected higher specificity, higher positive predictive value, and lower rate of inconclusive results.11,48 However, definitive results for the sensitivity and specificity of CNB as a follow-up study have not yet been obtained.30,41,47 CNB showed a lower sensitivity for thyroid glands than for other head and neck lesions.49 However, the initial test might be more important for achieving higher sensitivity and patient safety. Furthermore, CNB has some limitations, including bleeding, tumor-cell displacement, and difficulty in approaching thyroid nodules in a posterior portion or close to critical structures, including the carotid artery or trachea; these factors limit its use as an initial test.48,49 To obtain an adequate specimen for diagnosis, the experience of the operator may be more important for CNB than for FNA.48 Whether CNB is appropriate as an initial test for thyroid nodules is not fully understood and could not be determined in the current systematic review. Despite a previous report that found follow-up CNB to have lower non-diagnostic and inconclusive rates than rFNA (7.2% vs 72.0%),18 many previous studies have reported that CNB showed lower sensitivity than FNA. However, the diagnostic accuracy of follow-up CNB has not been fully elucidated. In the current DTA review, the pooled sensitivity and specificity of follow-up CNB were significantly high. For this reason, follow-up CNB after initial FNA might be useful for predicting malignancy and reducing the inconclusive rate in follow-up study.

There were some limitations in the current meta-analysis. First, most included studies were retrospective rather than prospective evaluations. Many thyroid nodules with initial inconclusive results only involved follow-up with ultrasonography. The conclusive and inconclusive rates of rFNA and follow-up CNB might have been affected by such cases. Therefore, cumu-

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Fig. 3. The summary receiver operating characteristics (SROC) curve of follow-up core needle biopsy for prediction of papillary thyroid carcinoma after prior fine needle aspiration with non-diagnostic or atypia/follicular lesion of undetermined significance in thyroid nodules. AUC, area under the curve.
ative prospective studies are needed to determine the effectiveness of follow-up procedures. Second, the current study included both non-diagnostic and AUS/FLUS subgroups, and subgroup analysis was performed. However, the DTA review could be not conducted for the rFNA subgroup due to insufficient information from eligible studies. Thus, a comparison of diagnostic accuracy between follow-up CNB and rFNA could not be performed. Third, because the current guidelines recommend initial FNA test for thyroid nodules, the current meta-analysis was performed only for studies with initial FNA. An investigation of the effectiveness of CNB as an initial test was therefore not conducted in the current study. Fourth, the current study was analyzed for initial non-diagnostic and AUS/FLUS categories. However, additional evaluations, including subgroup analysis for AUS and FLUS results, could not be performed due to lack of information on follow-up CNB from eligible studies. Fifth, eligible studies used various criteria for nondiagnostic or indeterminate lesion. The rate of inconclusive results of CNB may have differed from the real value. Further studies are needed to establish guidelines of pathology reporting for thyroid CNB.

In conclusion, the current study showed that follow-up CNB had a higher conclusive rate than rFNA after initial FNA with inconclusive results. In addition, follow-up CNB had greater diagnostic accuracy for prediction of malignancy than rFNA. Additional prospective studies are required to determine standardized application criteria of follow-up CNB in daily practice.

**Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.

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Investigation of the Roles of Cyclooxygenase-2 and Galectin-3 Expression in the Pathogenesis of Premenopausal Endometrial Polyps

Esin Kasap · Serap Karaarslan · Esra Bahar Gur · Mine Genc · Nur Sahin · Serkan Güçlü

Background: The pathogenesis and etiology of endometrial polyps has not been elucidated. In this study, we aimed to examine the pathogenic mechanisms of endometrial polyp development using immunohistochemistry. We evaluated the expression of galectin-3 and cyclooxygenase-2 (COX-2) during the menstrual cycle in premenopausal women with endometrial polyps or normal endometrium. Methods: Thirty-one patients with endometrial polyps and 50 healthy control patients were included in this study. The levels of expression of COX-2 and galectin-3 were studied by immunohistochemistry. Results: The percentage of COX-2–positive cells and the intensity of COX-2 staining in the endometrium did not vary during the menstrual cycle either in the control group or in patients with endometrial polyps. However, expression of galectin-3 was significantly lower in endometrial polyps and during the proliferative phase of the endometrium compared with the secretory phase. Conclusions: Our data suggests that the pathogenesis of endometrial polyps does not involve expression of COX-2 or galectin-3.

Key Words: Endometrial polyps; Cyclooxygenase-2; Galectin-3; Immunohistochemistry

Focal endometrial projections containing endometrial glands and stroma are termed endometrial polyps.1 The reported prevalence of endometrial polyps in premenopausal women with abnormal uterine bleeding is 33%, but only 10% in asymptomatic women.2 While endometrial polyps can occur in women of all ages, they are more common in women between the ages of 40 and 49.3 Because endometrial polyps have not been reported to occur before the onset of menstruation, estrogenic stimulation is thought to be associated with endometrial polyp growth.4 However, the pathogenesis and etiology of endometrial polyps has not been clearly determined. Endometrial polyps are believed to form due to hormonal factors, e.g., estrogen and progesterone, which mediate endometrial proliferation and differentiation via steroid receptors;5 however, the mechanisms involved in the development of endometrial polyps are still unclear.

Cyclooxygenase (COX) is a key enzyme involved in the conversion of arachidonic acid to prostaglandins and other eicosanoids. Two isoforms of COX have been identified: COX-1 and COX-2. COX-1 is constitutively expressed in many tissues, whereas COX-2 is induced by a variety of factors including cytokines, growth factors, and tumor promoters. COX-2, which is involved in inflammatory responses and production of prostanoids mediating uterine contractions, has been shown to induce excessive formation of some pro-angiogenic factors when overexpressed in colon cancer cell lines in vitro.6 Furthermore, recent studies have shown the influence of COX-2 in neoplastic development.5 However, the relationship between COX-2 and endometrial polyps has not been well established.

Galectin-3 is a β-galactoside–binding animal lectin that contains carbohydrate-recognition domains and is involved in a multitude of biological tasks,7 including embryogenesis, growth, cell adhesion, proliferation, differentiation, cell-cycle progression, apoptosis, mRNA splicing, and immune system regulation. Galectin-3 is also involved in tumorigenesis, angiogenesis, and tumor metastasis, and is expressed in various cells and tissues including activated macrophages, eosinophils, neutrophils, mast cells, gastrointestinal and respiratory tract epithelial cells, kidney cells, and some sensory neurons.7,8 Interestingly, intracellular galectin-3 plays a role in inflammation, while intracellular galectin-3 participates in cell growth and anti-apoptotic processes.
and modulates cell adhesion and migration.\(^7\)

Polyps tend to occur when apoptosis is inhibited and there is unopposed growth.\(^9\) However, the mechanisms mediating these endometrial alterations are unknown. Previous studies have suggested that endometrial polyps are a result of endometrial inflammation, i.e., endometritis, since the vessel axis of polyps has been shown to develop during endometritis.\(^10\) This finding suggests that identification of markers of inflammation and tissue growth may help to elucidate the pathogenic mechanisms of endometrial polyps. Indeed, recent studies have shown that the levels of expression of COX-2 and galectin-3 are increased during the progression from hyperplasia to cancer in the endometrial tissue, suggesting that these proteins may play a role in tumor cell function. However, the association of COX-2 and galectin-3 expression with polyps has not yet been established.

Therefore, in this study, we analyzed the levels of expression of COX-2 and galectin-3 in endometrial polyps and normal endometrium using immunohistochemistry.

**MATERIALS AND METHODS**

**Patients**

We examined a total of 81 cases of endometrial tissues in patients who were treated in the Department of Obstetrics and Gynecology of our hospital. Tissues were sampled between 2012 and 2014. All procedures were approved by the Izmir Sifa University Human Ethics Committee and followed the principles of the Declaration of Helsinki.

All patients were premenopausal (mean age, 37 years; range, 27 to 52 years) and had a recent history of regular menstruation (cycle of 25–35 days). None of the women included in the study used nonsteroidal anti-inflammatory drugs, hormone replacement therapy, or any other estrogen-containing pills. Thirty-one of the 81 patients had endometrial polyps, including 10 who had undergone hysterectomy and 21 who had undergone polypectomy and endometrial curettage. None of the patients had identifiable leiomyoma or adenomyosis by ultrasonography and/or magnetic resonance imaging. The control group consisted of samples from a total of 50 additional patients with normal endometrium, and included 23 samples collected during the proliferative phase and 27 samples collected during the secretory phase. Control patients were recruited from patients with benign ovarian cysts or a uterine prolapse but no other pathology, and the endometrial samples in this group were collected during hysterectomy procedures. Endometrial samples were grouped according to menstrual cycle phases: proliferative (days 1–14 of the cycle) and secretory (days 15–28 of the cycle). The day of the menstrual cycle was established from the women’s menstrual history and confirmed by endometrial dating using the criteria described by Noyes \textit{et al.}\(^11\)

**Immunohistochemistry**

All tissue samples were fixed in 10% formalin and sent to pathology for analysis. Routine hematoxylin and eosin staining was carried out in all samples either to confirm the diagnosis of polyps or to date the endometrium. Samples were embedded in paraffin blocks, cut into 4-μm-thick sections, and deparaffinized. The sections were then stained with primary monoclonal antibodies against COX-2 (1:100, clone CX-294, Dako, Glostrup, Denmark) and galectin-3 (1:100, NCL-GAL3, clone 9C4, NovoCastra, Hamburg, Germany) using a Dako Cytomation Autostainer (Dako). After staining, each sample was evaluated under a light microscope (200×, Olympus BX53, Olympus, Tokyo, Japan) to determine the percentage of COX-2–positive cells, the intensity of COX-2 staining, and the percentage of galectin-3–positive cells. For positive controls, staining of breast carcinoma tissue for COX-2 and papillary thyroid carcinoma tissue for galectin-3 were used. Primary monoclonal antibodies were omitted in negative controls.

**Assessment of COX-2 and galectin-3 staining**

Semi-quantitative analysis of immunostaining for COX-2 and galectin-3 was performed as follows based on the percentage of cells with positive cytoplasmic staining: 0%, 0; <10%, 1; 10%–50%, 2; 51%–80%, 3; and ≥80%, 4. In addition, staining intensity was evaluated as either negative (0), weak (1+), moderate (2+), or strong (3+). Semi-quantitative and intensity scores were analyzed separately. Additionally, the positivity of cells was evaluated as positive or negative.\(^12\) COX-2 and galectin-3 expression was evaluated in glandular epithelial cells and stromal cells. Assessment of staining results was performed by one observer in a blinded fashion.

**Statistical analysis**

Statistical analysis was performed using software (Rstudio software ver. 0.98.501 via R language, R Studio Inc., Boston, MA, USA). Data describing continuous variables are presented as the mean ± standard deviation. The Kruskal-Wallis and Pearson chi-square exact tests were used to compare continuous and categorical variables, respectively. Differences with p-values less than .05 were accepted as significant.
RESULTS

Patient demographics

There was no statistically significant difference between the ages of healthy control individuals and patients with endometrial polyps.

COX-2 expression

Immunoreactivity for COX-2 was observed in glandular epithelial cells and stromal cells. COX-2–positive cells were predominantly observed in the endometrial glandular epithelium, where expression peaked during the secretory phase. COX-2–positive cells were also observed in stromal cells, albeit to a lesser extent (Table 1, Fig. 1A). The percentage of COX-2–positive cells and the intensity of COX-2 staining in stromal cells and glandular epithelial cells did not vary during different periods of the menstrual cycle in the control group or in patients with endometrial polyps (Fig. 1B). Mean COX-2 scores in glandular epithelial cells and stromal cells were not significantly different between endometrial polyp specimens and normal endometrium specimens (Table 1).

Galectin-3 expression

Galectin-3 immunoreactivity was present in the endometrial glandular epithelial cells and stromal cells. Immunostaining was typically cytoplasmic. Galectin-3–positive cells were predominantly observed in the endometrial glandular epithelium, where expression levels peaked during the secretory phase. Galectin-3 expression was also observed to a lesser extent in stromal cells (Table 2). The mean percentage score of galectin-3 expression were lower both in endometrial polyps and the proliferative phase in normal endometrium than in the secretory phase in normal endometrium (Table 2, Fig. 1C). In glandular epithelial cells, no statistically significant differences in galectin-3 expression were found between endometrial polyps and normal endometrium during the proliferative phase. However, in patients with normal endometrium, galectin-3 expression was higher during the secretory phase (p = .349) (Fig. 1C). Finally, there

Table 1. Percentages of COX-2–positive cells and intensity of COX-2 staining

|                      | Proliferative phase | Secretory phase | Endometrial polyps | p-value |
|----------------------|---------------------|-----------------|--------------------|---------|
| Glandular epithelial tissues |                    |                 |                    |         |
| COX-2 intensity      | 2.04 ± 1.02         | 2.07 ± 0.83     | 1.77 ± 1.11        | .489a   |
| 0–1                  | 7                   | 6               | 12                 | .398b   |
| 2–3                  | 16                  | 21              | 19                 |         |
| COX-2 percentage score | 2.35 ± 1.12         | 2.67 ± 1.04     | 2.35 ± 1.38        | .614c   |
| 0–1                  | 4                   | 3               | 8                  | .351d   |
| 2–4                  | 19                  | 24              | 23                 |         |
| Endometrial stroma   | COX-2 intensity     |                 |                    |         |
| 0–1                  | 0.65 ± 0.71         | 0.70 ± 0.67     | 0.53 ± 0.68        | .564e   |
| 0–3                  | 20                  | 24              | 27                 | .941f   |
| 2–4                  | 3                   | 3               | 3                  |         |
| COX-2 percentage score | 0.61 ± 0.65         | 0.81 ± 0.78     | 0.61 ± 0.76        | .525g   |
| 0–1                  | 21                  | 21              | 26                 | .430h   |
| 2–4                  | 6                   | 6               | 5                  |         |

Values are presented as mean ± standard deviation.
COX, cyclooxygenase.
*p-value for Kruskal-Wallis tests, *p-value for Pearson chi-square test.

Fig. 1. Expression of cyclooxygenase 2 (COX-2) and galectin-3 in endometrium samples. (A) COX-2 expression in both the glandular epithelium and stroma during the secretory phase. (B) COX-2 expression in both the glandular epithelium and stroma of an endometrial polyp. (C) Galectin-3 expression in both the glandular epithelium and stroma during the secretory phase.
were no differences in galectin-3 expression in stromal cells of endometrial polyps and those of the endometrium at any phase of the menstrual cycle (Table 2).

**DISCUSSION**

In this study, we compared galectin-3 and COX-2 expression and staining patterns using immunohistochemistry in endometrial polyps and normal endometrium during the secretory and proliferative phases. Our data indicated that both galectin-3 and COX-2 were not associated with the formation of endometrial polyps.

Estrogen is known to play a pivotal role in the pathogenesis of endometrial malignant and benign cancers. However, the role of estrogen biosynthesis from stromal cells of the endometrium and its impact on malignancies has not been fully elucidated. Interestingly, estrogens are known to activate COX-2 in endometriosis, which increases levels of estradiol and prostaglandin above normal in women. Therefore, the pathogenesis of peritoneal endometriosis may be a consequence of enhanced prostaglandins in the eutopic endometrium that stimulate COX-2 expression and activity. This mechanism may also affect other endometrial pathologies, although no specific roles have been described.

COX-2 is expressed at higher levels in the glandular epithelial cells of the endometrium during the secretory phase of the menstrual cycle compared with the proliferative phase; however, its expression is similar during the secretory and proliferative phases in endometrial stromal cells. In the present study, we observed that COX-2 expression tended to be higher during the secretory phase than in either the proliferative phase or endometrial polyps in glandular epithelial cells.

Some studies have suggested that endometrial polyps may originate from endometrial inflammation. After demonstrating that COX-2 is expressed in the epithelial lining of postmenopausal polyps, Maia et al. concluded that COX-2 may be involved in the regulation of polyp growth. Nevertheless, COX-2 expression has not been shown to differ significantly between normal endometrial tissue and endometrial polyps.

In 2006, Maia and colleagues compared COX-2 expression between endometrial polyps and normal endometrium in women with a regular menstrual cycle and found no significant difference in COX-2 expression between polyps and the normal endometrium. In addition, Pinheiro et al. observed higher COX-2 expression in the glandular epithelium of obese women than in women of normal weight. This finding indicates that expression of COX-2 expression may be influenced by metabolic changes and growth factors associated with obesity. Here, we also demonstrated that COX-2 expression in the glandular epithelium and stromal cells in endometrial polyps did not differ significantly from that of normal endometrium. Thus, our data suggest that COX-2 expression may not be associated with the formation of endometrial polyps.

We previously reported that expression of galectin-3 is augmented during the formation of the receptive endometrium and the mid-secretory phase of the menstrual cycle. In addition, there is mounting evidence for a strong relationship between embryo implantation and tumor invasion and metastasis. Interestingly, galectin-3 has been reported to have anti-apoptotic potential in tumor cells, although there is no evidence for the role of galectin-3 in endometrial cell apoptosis. Thus, we hypothesize that galectin-3 functions as an anti-apoptotic factor in the endometrium and may facilitate the development of endometrial polyps.

Chiu et al. found that immunohistochemical analysis of galectin-3 protein expression is a sensitive, specific, and accurate marker for the diagnosis of thyroid cancer and certain other cancers. In endometriosis, Noel et al. found that galectin-3 protein expression, as measured using immunohistochemistry, is higher in endometriosis samples compared to eutopic endometrium, and also higher in the eutopic endometrium of women with endometriosis compared to those without endometriosis. Together, these data suggest that galectin-3 may have a potential role in the development of endometriosis. In a study by Brustmann et al., galectin-3 expression was assessed by immunohistochemistry in patients with normal endometrium, simple hyperplasia, complex hyperplasia without atypia, atypical hyperplasia, endometrioid carcinoma, serous papillary carcinoma, and clear cell carcinoma. They showed that in normal endometrium, simple hyperplasia, and complex hyperplasia without atyp-
ia, the galectin-3 expression did not differ significantly among these groups. Immunohistochemical expression of galectin-3 for atypical hyperplasia and endometrioid carcinoma were significantly higher than those in the aforementioned groups. Serous papillary carcinoma and clear cell carcinoma, which are known to have a poorer prognosis than endometrioid carcinoma, also demonstrated significantly elevated galectin-3 expression levels in this study. On the basis of these findings, we assumed that galectin-3 expression may be related to the different biological behaviors of endometrial carcinomas. Importantly, while there was no difference in galectin-3 expression in glandular epithelium and stromal cells of endometrial polyps and normal endometrium, our data demonstrated that galectin-3 expression was significantly lower in both endometrial polyps and the proliferative phase of the endometrium than in the secretory phase of the endometrium. This finding suggests that larger-scale studies are needed to measure galectin-3 levels in secretory adenocarcinomas, a subgroup of endometrioid adenocarcinomas, and in other endometrial cancers.

In analyzing our results, we wondered whether galectin-3 expression may be regulated by estrogen and progesterone. Although previous reports have shown that estrogen and/or progesterone increases galectin-3 expression in endometrial cells at all concentrations, we did not observe any direct effect of these hormones on galectin-3 expression, suggesting that the relationship may be indirect. Thus, further studies are needed to determine the exact nature of the relationship of estrogen and progesterone on galectin-3 expression.

The small sample size was a limitation of the present study. Therefore, the results of this study must be confirmed in larger longitudinal population studies.

In summary, our data suggested that endometrial polyps may form through certain common pathways that do not involve COX-2 and galectin-3. While COX-2 and galectin-3, in addition to angiogenesis, are known to be involved in different steps of carcinogenesis, these two target proteins may not be involved in the formation of endometrial polyps. Further large scale studies comparing adjacent endometrial tissue and healthy women at specific phases of the menstrual cycle should be performed.

**Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.

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Rare Case of Anal Canal Signet Ring Cell Carcinoma Associated with Perianal and Vulvar Pagetoid Spread

Na Rae Kim · Hyun Yee Cho
Jeong-Heum Back · Juhyeon Jeong
Seung Won Park · Sun Jin Sym
Kyu Chan Lee · Dong Hae Chung

Departments of Pathology and General Surgery, Division of Hematology and Oncology, Department of Internal Medicine, Department of Radiation Oncology, Gachon University Gil Medical Center, Incheon, Korea

A 61-year-old woman was referred to surgery for incidentally found colonic polyps during a health examination. Physical examination revealed widespread eczematous skin lesion without pruritus in the perianal and vulvar area. Abdominopelvic computed tomography showed an approximately 4-cm-sized, soft tissue lesion in the right perianal area. Inguinal lymph node dissection and Mils’ operation extended to perianal and perivulvar skin was performed. Histologically, the anal canal lesion was composed of mucin-containing signet ring cells, which were similar to those found in Pagetoid skin lesions. It was diagnosed as an anal canal signet ring cell carcinoma (SRCC) with perianal and vulvar Pagetoid spread and bilateral inguinal lymph node metastasis. Anal canal SRCC is rare, and the current case is the third reported case in the English literature. Seven additional cases were retrieved from the world literature. Here, we describe this rare case of anal canal SRCC with perianal Pagetoid spread and provide a literature review.

Key Words: Carcinoma, signet ring cell; Anal canal; Paget disease, extramammary

According to the recent World Health Organization classification of the digestive system, patients with mucinous adenocarcinoma have a significantly high survival rate compared to those with signet ring cell carcinoma (SRCC). By definition, SRCC has more than 50% intracellular mucin and mucinous carcinoma has more than 50% extracellular mucin. SRCC can arise from virtually all organs, including stomach, colon, breasts, gallbladder, or even urinary bladder. When an extragastric SRCC is found in an unusual site, metastasis from other primary sites should be excluded. Among colorectal SRCCs of the digestive tract, anal canal SRCCs are even rarer. Only two cases of anal canal SRCC have been described in the English literature. We recently encountered a rare case of anal canal SRCC and extramammary Paget’s disease. Extramammary Paget’s disease commonly appears in the vulvar area, followed by the perianal region, scrotum, penis, and axillae. In most cases, Paget’s disease is not accompanied by cancer; however, cancer hidden in the adjacent organs, including the vulva, vagina, uterus, ovary, bladder, and colorectal area, should be kept in mind.

Here, we report on a case of anal canal SRCC and Pagetoid spread, and provide a literature review.

CASE REPORT

A 61-year-old woman visited our hospital for alleged colon polyps found during a colonoscopy examination as part of a routine health examination. Endoscopic submucosal dissection was performed. Four polyps were diagnosed as tubular adenoma. Abdominopelvic computed tomography (CT) showed an approximately 4-cm-sized, well-enhancing soft tissue lesion in the right perianal area (Fig. 1A) and a well-enhancing enlarged lymph node in both inguinal areas, suggesting hypervascular tumor metastasis such as melanoma in those lymph nodes (Fig. 1B). Upon physical examination, however, a widespread ery-
thematous lichenified skin lesion, measuring 12 × 9.7 cm, was noted in the perianal and vulvar area (Fig. 2A), which had developed over the previous 3 years according to the patient. She did not complain of pruritus. She had no history of anal fissure, fistula, or change in bowel habit. Skin biopsy was taken from the perianal and vulvar area, which showed infiltrating Pagetoid cells and SRCC cells. There was neither abnormal bowel wall thickening nor masses in the sigmoid colon. No other lesion was observed on upper esophagogastroscopy. The preoperative carcinoembryonic antigen (CEA) level was elevated to 39.75 ng/mL (reference, 0 to 5 ng/mL). Carbohydrate antigen 19-9 was within normal ranges. Inguinal lymph node dissection with an extended Miles’ operation was performed; traditional Miles’ operation with extended resection around bilateral labium majus, followed by skin reconstruction with gluteal flap, was performed. The resected specimen was composed of the anal canal and rectum with skin and soft tissue of the perianal region and vulva (Fig. 2B). Upon sectioning, the anal canal showed an ulcerative firm mass measuring 4.0 × 3.0 × 2.7 cm, which invaded the perianal sphincter muscle and subcutaneous fat of the anal skin (Fig. 2C). Light microscopy showed that the anal canal was totally replaced by singly scattered intracytoplasmic mucin-containing signet ring cells and some extracellular mucin (Fig. 3A, B). Focally, well-formed glands were also found in less than 5% of the tumor. Most of the surface epithelium was denuded. The focal residual transformation zone showed a focus of adenocarcinoma in situ (Fig. 3C), and was not contiguous with the anal glands. Anal canal SRCC was diagnosed. The erythematous skin around the anus and labium majus showed linear infiltration of Pagetoid cells as well as infiltration of signet ring cells in the dermis and subcutis (Fig. 3D). Involved skin area measured 8.2 × 8.0 × 0.5 cm. Lymphatic permeation was noted in the dermis. Enlarged bilateral inguinal lymph nodes also showed infiltration of the signet ring cells.

Immunohistochemically, both signet ring cells and Pagetoid cells were positive for cytokeratin (CK) 20, CEA, MOC-31, and CK19. Intracellular and extracellular mucin was eosinophilically stained with mucicarmine. Both signet ring cells and Paget’s

Fig. 1. Radiologic findings. (A) Abdominal computed tomography reveals a well-enhancing lesion (arrow) at the right perianal portion. (B) Enlarged bilateral inguinal lymph nodes (arrows) are found.

Fig. 2. Gross pictures. (A) Perianal and vulvar skin shows elevated erythematous changes. (B) Extended Miles’ operation specimen consists of distal rectal segment and excision of perianal region and vulva. (C) Cross-section of the resected specimen shows a firm anal canal mass infiltrating to the perianal skin and levator ani muscle.
cells were positive for epidermal growth factor receptor and weakly positive for CK5/6. They were negative for CK7, human melanoma black 45, S-100 protein, caudal-related homeobox gene nuclear transcription factor (CDX2), p53, synaptophysin, chromogranin, and CD56. These results are shown in Table 1.

A mutation study of the KRAS gene (codon 12 and codon 13) was performed by means of the peptide nucleic acid-mediated real-time polymerase chain reaction clamping method using genomic DNA isolated from formalin fixed paraffin-embedded tissue. Mutations in codon 12 and 13 were not detected in anal canal SRCC.

Microsatellite instability (MSI) was tested using five Bethesda markers (D2S123, D5S346, D17S250, BAT25, and BAT26). MSI-high (MSI-H) was defined if they differed in at least two of the five markers and MSI-low was defined if they differed in only one of the five markers. Anal canal mass was microsatellite stable (MSS).

Positron emission tomography CT and chest CT showed no evidence of systemic metastases. The case was diagnosed as anal canal SRCC with Pagetoid spread in perianal and vulva skin, stage IIIB according to the TNM staging system of the Ameri-
can Joint Committee on Cancer.

The patient was scheduled to undergo chemotherapy with mitomycin-C and 5-fluorouracil (5-FU) and subsequent radiotherapy. The patient’s postoperative course was unremarkable. Institutional review board (IRB) approval was obtained for this case report.

**DISCUSSION**

Colorectal SRCC is a rare histologic subtype of adenocarcinoma, accounting for 0.1% to 2.4% of all colorectal malignancies.\(^1\) Anal canal SRCC is even rarer; only two cases of anal canal SRCCs have been reported in the English literature.\(^1\) Seven additional cases have been retrieved from the Japanese literature.\(^3-5,7-12\) The current case showed anal canal SRCC. Anal canal carcinoma is histologically and pathogenetically divided into squamous cell carcinoma, cloacogenic (basaloid or transitional cell) carcinoma and adenocarcinoma.\(^13\) Anal canal squamous cell carcinoma originates from the non-keratinizing squamous epithelium below the dentate line of the anal canal, while anal canal adenocarcinoma is regarded as arising from the upper part lined by the columnar epithelium. Anal canal adenocarcinomas are rare. According to the World Health Organization classification, anal canal adenocarcinomas are subclassified according to adenocarcinoma arising from anal mucosa and extramucosal adenocarcinoma of anal glands.\(^14\) No description of in situ lesion was found in the previously reported cases of anal canal SRCCs. Based on the findings of the overlying ulcerated anal surface mucosa, remnants of anal gland adenocarcinoma in situ without continuity to SRCC portion, the present anal canal SRCC belongs to extramucosal perianal adenocarcinoma with wide Pagetoid spread along the perianal soft tissues and skin.

We have summarized the clinicopathologic characteristics in Table 2. Similar to the current case, eight out of 10 anal canal SRCCs (80%) were accompanied by perianal or vulvar Pagetoid spread.\(^3-5,7-12\) Extramammary perianal Paget’s disease is a rare and heterogeneous neoplasm, which is frequently combined with underlying hidden adenocarcinoma.\(^6\) Immunohistochemical panels such as CK7, CK20, CEA, gross cystic disease fluid protein-15 (GCDFP-15), MUC1, MUC2, MUC5AC, and CDX2 have been used to distinguish primary from secondary Paget’s disease.\(^15\) In anal SRCCs with Pagetoid spread, i.e., not primary extramammary Paget’s disease, the tumor cells were positive for CK20, MUC1, or MOC-31, while they were negative for GCDFP-15, and negative for CK7, whereas primary Paget’s disease is commonly positive for CK7.\(^16\) However, none of these were a specific and confirmative diagnostic clue; further accumulative data may be needed. SRCC is most commonly encountered in the stomach, but it can also arise in various organs, such as breast, urinary bladder, or colon. Immunohistochemical profiles for CK7 and CK20 have also been used to differentiate the sites of origin for SRCCs; CK20 is normally expressed in the gastrointestinal epithelium, urothelium, and in Merkel’s cells. While CK7 is uncommonly expressed in the lower gastrointestinal tract, it is commonly expressed in lung, ovary, endometrium, and breast.\(^15\)

| Clone, dilution, company | Anal canal signet ring cell carcinoma | Pagetoid spread of vulvar and perianal skin |
|--------------------------|-------------------------------------|------------------------------------------|
| CK7 OV-TL 12/30; 1:100 dilution; Dako, Glostrup, Denmark | − | − |
| CK20 Ks20.8; 1:100 dilution; Dako, Glostrup, Denmark | + | + |
| CK19 A53-B/A2.26; 1:200 dilution; Thermo Scientific, Fremont, USA | + | + |
| CK5/6 D5/6 B4; prediluted; Dako, Glostrup, Denmark | + weak | − |
| EGFR SP84; 1:30 dilution; Novocastra, Newcastle upon Tyne, UK | + | + |
| CEA Polyvalonal; prediluted; Dako, Glostrup, Denmark | + | + |
| MOC-31 MOC-31; 1:70 dilution; Novocastra, Newcastle upon Tyne, UK | + | + |
| CDX2 AMT28; 1:50 dilution; Novocastra, Newcastle upon Tyne, UK | − | − |
| S-100 protein Polyvalonal; 1:1,200 dilution; Dako, Glostrup, Denmark | − | − |
| Synaptophysin DAK-SYNAP; prediluted; Dako, Glostrup, Denmark | − | − |
| Chromogranin polyvalonal; prediluted; Dako, Glostrup, Denmark | − | − |
| CD56 12C3; prediluted; Dako, Glostrup, Denmark | − | − |
| HMB45 HMB45; prediluted; Dako, Glostrup, Denmark | − | − |
| p53 DO-7; 1:100 dilution; Dako, Glostrup, Denmark | + | + |

CK, cytokeratin; EGFR, epidermal growth factor receptor; CEA, carcinoembryonic antigen; CDX2, caudal-related homeobox gene nuclear transcription factor; HMB45, human melanoma black 45.
### Table 2. Clinicopathologic summary of the reported cases of primary anal canal signet ring cell carcinomas with or without Paget’s disease

| Case No. | Author | Age (yr)/Sex | Presenting symptom | Gross and histopathology | Immunohistochemistry | TNM at the initial evaluation | Treatment | Clinical outcome (follow-up period) |
|----------|--------|-------------|--------------------|-------------------------|----------------------|-----------------------------|-----------|------------------------------------|
| 1        | Uchigasaki et al. (1992) | 35/F | Anal ulcer and pruritus for 2 yr | Anal canal SROCC progressed from adenocarcinoma in situ of anal glands or mucinous glands (1.5 cm), Pagetoid spread (7 cm) | pCEA+, mCEA+, mucicarmine+, PAS+, D-PAS-resistant | T1N3M0, stage IIIB | Topical 5-FU cream, EB, ASR | Recurrence with systemic metastasis and died (7 mo) |
| 2        | Morihisa et al. (1999) | 75/F | Perianal erythema and erosion for 7 mo | Anal canal SROCC (size not described), Pagetoid spread (5 cm) | CEA+, mucicarmine+, PAS+, N3M1, stage IV | Nephrostomy for ARF due to systemic metastasis | Systemic metastasis and died (4 mo) |
| 3        | Nagano et al. (1999) | 46/F | Inguinal LN enlargement | Anal canal and rectal SROCC (3.5 cm), Pagetoid spread (7.5 cm) | Alcian blue+, PAS+, N3M1, stage IV | APR, CTX (5-FU, LV) | NR (3 mo) |
| 4        | Naganuma et al. (2004) | 85/F | Anal bleeding for 3 yr | Anal canal SROCC (unkown size of polyp, residual anal canal mass, 5 mm), Pagetoid spread (size not described) | CEA+, CA19-9+, CK20+, CK7+, GCDFP-15+, N3M1, stage IV | Anal polypectomy with CTX (5-FU), RT | Systemic metastasis and died (25 mo) |
| 5        | Nishimura et al. (2004) | 76/F | Anal erosion and erythema for 2 yr | Anal canal SROCC (size not described), Pagetoid spread (6 cm) | CK20+, GCDFP-15+, Not available | APR, CTX (5-FU, cisplatin), CTX (5-FU, LV) | NR (5 mo) |
| 6        | Yoshinari et al. (2009) | 50/M | Axillary and inguinal LN enlargement for 1 mo, erythematous perianal skin | Anal canal SROCC (size not precisely described; anal verge to dentate line), Pagetoid spread (size not described) | OX20+, MUC-1+, CK7+, N3M1, stage IV | CTX (bevacizumab, mFOLFOX6, FOLFIRI), APR, CTX (5-FU, LV, mFOLFOX6) | Alive with CR evaluated by PET-CT, CTX (bevacizumab, mFOLFOX6, FOLFIRI) | |
| 7        | Ikezawa et al. (2010) | 53/M | Anal pruritus for 22 mo | Anal canal SROCC (1 cm), Pagetoid spread (2 cm) | pCEA+, CK20+, CK7+, GCDFP-15+, T1N2M0, stage IIIB | APR, CTX (5-FU, LV), RT | Systemic metastasis and died (9 mo) |
| 8        | Ioannidis et al. (2012) | 87/F | Anal discomfort and pain for 8 mo | Anal canal SROCC (3 cm) | Not done | Wide excision with chemotherapy (5-FU) and RT | NR (6 yr) |
| 9        | Terada (2014) | 49/M | Anal bleeding | Anal canal SROCC (<2 cm) | AE1/3+, CK8+, CK19+, CEA+, CA19-9+, synaptophysin+, CDX2+, MUC1+, MUC2+, EMA+, CK7+, CK20+, CK5/6+, MUC4+, MUC6+, CD56+, NSE+, chromogranin+, vimentin- | T1N1M0, stage IIIA | Miles' operation with LN dissection | Metastatic carcinomatosis and died (5 mo) |
| 10       | Kim et al. (2016) | 61/F | Erythematous perianal and vulvar skin for 3 yr, synchronous colon adenocarcinoma (8 cm) | Anal canal SROCC (4 cm), Pagetoid spread (8.2 cm) | pCEA+, CK20+, CK19+, MOC-31+, CDX2+, CK7+, HMB45+, mucicarmine- | T2N3M0, stage IIIB | Extended Miles' operation and inguinal LN dissection, CTX (5-FU, MTC), RT (planned) | NR (2 mo) |

F, female; SROCC, signet ring cell carcinoma; pCEA, polyclonal carcinoembryonic antigen; mCEA, monoclonal carcinoembryonic antigen; PAS, periodic acid-Schiff; D-PAS, PAS diastase stain; 5-FU, 5-fluouracil; EB, electron beam therapy; ASR, abdominosacral resection; CEA, carcinoembryonic antigen; ARF, acute renal failure; LN, lymph node; APR, abdominosacral resection; CTX, chemotherapy; LV, leucovorin; NR, no recurrence; CA19-9, carbohydrate antigen 19-9; CK, cytokeratin; GCDFP-15, gross cystic disease fluid protein-15; RT, radiotherapy; M, male; mFOLFOX6, leucovorin, 5-FU, and oxaliplatin; FOLFIRI, irinotecan, 5-FU, and leucovorin; CR, complete remission; PET-CT, positron emission tomography-computed tomography; EMA, epithelial membrane antigen; NSE, neuron specific enolase; HMB-45, human melanoma black 45; MTC, mitomycin-C.

*aThis case was diagnosed as Paget’s disease 15 years before the anal canal SROCC; *bThese articles were not available.
As the term "signetring cells" implies, the tumor cells have intracellular mucin vacuoles displacing the nucleus to one side of the cells scattered as single cells or in loose clusters. This is caused by disrupted cell-to-cell adhesion and diffuse spreading, and results in more frequent lymphovascular invasion and node metastasis of SRCC as compared with other mucinous adenocarcinoma.

In view of the clinical aspects, colorectal SRCC has a very high mortality rate compared to mucinous carcinoma and non-mucinous colorectal adenocarcinomas. Even a minor signet-ring cell component, i.e., 50% or less, in colorectal carcinomas was independently associated with a high mortality rate, regardless of molecular or other clinicopathological factors. Anal canal SRCC grows insidiously underneath the mucosa, and thus it may not be found before the late advanced stage. It is uncertain whether anal SRCCs have an adverse prognosis like that of colorectal SRCC. Inguinal nodes should be examined for anal cancer staging. In a review of 10 cases of anal canal SRCCs, the ages ranged from 46 to 87 years (mean, 63.4 years). Two cases were proven as primary anal canal SRCCs by autopsy. SRCC has a shorter patient survival. During the follow-up period, ranging from 4 to 25 months, death caused by lymphatic or peritoneal carcinomatosis or distant metastases occurred in 50% of cases (5/10). The five remaining cases were alive with a follow-up period ranging from 2 months to 6 years. None of the previous cases underwent molecuclocytogenetic application. For the past decade, molecular prognostic markers in colorectal carcinoma have been studied; MSI-H is a well-established reliable predictive marker for chemotherapeutic efficacy in colorectal carcinoma. However, contrary to colorectal SRCCs that take an aggressive course, colorectal mucinous carcinoma takes a favorable course, although both are associated with MSI-H. Data on anal canal SRCC concerning MSI-H as a predictive factor is lacking. The current case showed MSS, albeit it is only one case. Due to the rarity of anal canal SRCCs, the MSI status of anal canal SRCC relating to prognosis has not been determined. Further accumulative investigation is required.

Traditional treatment of anal canal carcinoma is surgery including abdominopelvic resection with or without chemoradiotherapy. Radiation therapy for anal canal carcinoma reaches a cure rate of up to 70% in selected low-stage patients. However, there is no general agreement regarding the treatment of anal canal SRCC due to the extremely rare incidence and limited data on cancer treatment, as shown in Table 2. In a case reported by Yoshitani et al., a patient with distant metastatic foci achieved complete remission after chemotherapy using bevacizumab/mFOLFOX6, FOLFIRI. Ioannidis et al. reported on a long-survived stage II patient with no recurrent tumor who underwent chemoradiotherapy using 5-FU for a 6-year follow-up period.

In summary, anal canal SRCC is extremely rare. If there is perianal Paget’s disease raising the possibility of underlying hidden adenocarcinoma, careful evaluation is necessary for early diagnosis of anal canal SRCC.

**Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.

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Sclerosing Perivascular Epithelioid Cell Tumor of the Lung: A Case Report with Cytologic Findings

Ha Yeon Kim · Jin Hyuk Choi
Hye Seung Lee · Yoo Jin Choi
Aeree Kim · Han Kyeom Kim

Department of Pathology, Korea University Guro Hospital, Seoul, Korea

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Corresponding Author
Han Kyeom Kim, MD, PhD
Department of Pathology, Korea University Guro Hospital, 148 Gurodong-ro, Guro-gu, Seoul 08308, Korea
Tel: +82-2-2626-3251
Fax: +82-2-2626-1481
E-mail: sswords@naver.com

Benign perivascular epithelioid cell tumor (PEComa) of the lung is a rare benign neoplasm, a sclerosing variant of which is even rarer. We present a case of 51-year-old man who was diagnosed with benign sclerosing PEComa by percutaneous fine needle aspiration cytology and biopsy. The aspirate revealed a few cell clusters composed of bland-looking polygonal or spindle cells with fine granular or clear cytoplasm. Occasional fine vessel-like structures with surrounding hyalinized materials were seen. The patient later underwent wedge resection of the lung. The histopathological study of the resected specimen revealed sheets of polygonal cells with clear vacuolated cytoplasm, variably sized thin blood vessels, and densely hyalinized stroma. In immunohistochemical studies, reactivity of tumor cells for human melanoma black 45 and Melan-A further supported the diagnosis of benign sclerosing PEComa. To the best of our knowledge, this is the first case of benign sclerosing PEComa described in lung.

Key Words: Lung neoplasms; Solitary pulmonary nodule; Perivascular epithelioid cell neoplasms

CASE REPORT

We report a case of a 51-year-old male patient diagnosed with benign PEComa by percutaneous fine needle aspiration of the lung. He had hypertension, diabetes mellitus and history of smoking (30 pack-years). He complained no specific symptoms. On chest X-ray, a well-demarcated mass was incidentally found in the periphery of the right upper lobe. It measured 1 cm and was confined to lung parenchyma.

Computed tomography-guided percutaneous aspiration and gun biopsy was simultaneously performed. The lesion was diagnosed as benign sclerosing PEComa on the basis of cytologic and histologic findings. After the diagnosis, the patient underwent subsequent wedge resection of the lung. The histologic and immunohistochemical findings of the resected specimen confirmed the diagnosis of benign sclerosing PEComa.

Cytologic findings

Alcohol-fixed liquid-based preparation (ThinPrep, Cytyc Corporation, Boxborough, MA, USA) was performed after percutaneous fine needle aspiration. The cytologic preparation was hypocellular with a few cell clusters in a clean background. These clusters consisted of polygonal to spindle cells with oval nuclei and small distinct nucleoli. Most of the cells showed abundant basophilic and granular cytoplasm or clear intracytoplasmic vac-
uoiles (Fig. 1A). Nuclear pleomorphism was minimal. Neither necrosis nor mitotic figures was observed. Some of the clusters had thin vessel-like structures within (Fig. 1B). Semitranslucent hyalinizing material was noted around the vascular structures (Fig. 1C).

**Histologic findings of biopsied specimen**

Gun-biopsied specimen was also composed of bland-looking polygonal cells with vacuolated cytoplasm and minimal atypia. Variable-sized vascular spaces were observed among sheets of tumor cells. Dilated vessels were surrounded by hyalinized stroma. Focal microcalcification was noted. Tumor cells were positive for human melanoma black 45 (HMB-45). Intracytoplasmic vacuoles were confirmed as glycogen by periodic acid–Schiff stain and diastase periodic acid–Schiff stain.

**Gross findings of resected specimen**

Wedge resected specimen of the right upper lobe revealed a 1.0-cm-sized, well-demarcated, non-encapsulated, and round mass. It was well confined within the pulmonary parenchyma. The mass was tan-colored and had a firm texture. The cut surface of the mass showed multiple tiny cyst-like spaces at the periphery.

**Histologic findings of resected specimen**

On light microscopy, the histologic findings of the surgical specimen were similar to those of the gun-biopsied specimen. The tumor was mainly composed of sheets of polygonal cells with distinct cell borders. Tumor cells showed clear cytoplasmic vacuoles and round to oval nuclei with granular chromatin and distinct nucleoli (Fig. 2A). Numerous dilated, thin walled blood vessels were present throughout the tumor. Those vessels were surrounded by pinkish hyalinized stroma (Fig. 2B). A few microcalcifications were seen. The cytoplasmic vacuoles were revealed to be glycogen on periodic acid–Schiff stain and diastase periodic acid–Schiff stain (Fig. 3A, B). Those vacuoles were not

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Fig. 1. Liquid-based aspiration cytology of benign sclerosing perivascular epithelioid cell tumor. (A) A few cohesive clusters of polygonal to spindle-shaped bland cells in a clean background (Papanicolaou staining). (B) The tumor cells show oval nuclei, small distinct nucleoli and abundant basophilic and granular cytoplasm. Most cells contain clear intracytoplasmic vacuoles (Papanicolaou staining). (C) Thin-walled blood vessels are occasionally seen. Semitranslucent hyalinizing material was noted around the vascular structure (Papanicolaou staining).

Fig. 2. Histologic section of benign sclerosing perivascular epithelioid tumor. (A) Dilated vessels surrounded by collagenous stroma and polygonal cells with clear cytoplasm. (B) Sheet-like arrangement of tumor cells around various-sized blood vessels and a focus of microcalcification.
reactive in mucicarmine and alcian blue stainings.

**Immunohistochemical findings**

Various immunohistochemical studies were performed. The results are summarized in Table 1. Tumor cells showed positivity for HMB-45 and Melan-A (Fig. 3C, D). They were negative for S-100 protein, neuroendocrine markers (CD56, synaptophysin, and chromogranin), pulmonary adenocarcinoma markers (thyroid transcription factor 1 [TTF-1], surfactant, and napsin), and cytokeratins. Ki-67 labeling index was low (< 2%).

**DISCUSSION**

Benign PEComa of the lung was first described in 1963 by Leiwbow and Castleman. It was termed “sugar tumor” due to the intracytoplasmic glycogen content. It is a rare benign neoplasm and about 50 cases have been reported in the English literature so far.

| Test                  | Result                  |
|-----------------------|-------------------------|
| HMB-45                | Positive                |
| Melan-A               | Positive                |
| S-100                 | Negative                |
| CD56                  | Negative                |
| Synaptophysin         | Negative                |
| Chromogranin          | Negative                |
| Vimentin              | Positive                |
| TTF-1                 | Negative                |
| Surfactant            | Negative                |
| Napsin                | Negative                |
| Cytokeratin           | Negative                |
| p63                   | Negative                |
| Ki-67                 | Positivity in <2%       |
| CD68                  | Negative                |
| SMA                   | Positive of intratumoral vasculature |
| CD34                  | Positive of intratumoral vasculature |

HMB-45, human melanoma black 45; TTF-1, thyroid transcription factor 1; SMA, smooth muscle antigen.
Patients with benign PEComas of the lung are generally asymptomatic. Our case was similar to most other cases in that the patient had no symptoms and the tumor was detected incidentally. A few cases presented with hemoptysis. One case presented with thrombocytosis which resolved after the removal of the tumor.

Typical aspirate specimens of benign PEComas show loosely cohesive clusters of bland-looking cells of various sizes. The cells are mostly epithelioid or spindle and have round-to-oval nuclei, indistinct nucleoli, and vacuolated cytoplasm. Nuclear atypia is rare and necrosis is not observed. Our findings were generally similar to the previous cytologic reports of Nguyen in 1989, Edelweiss et al. in 2007, and Policarpio-Nicolas et al. in 2008. Two exceptional findings include small distinct nucleoli and fine granular basophilic cytoplasm, while others reported indistinct or inconspicuous nucleoli and clear, vacuolated cytoplasm. Different preparation methods might have contributed to these differences. In our case, the aspiration specimen was fixed in alcohol and prepared according to the liquid-based method. Other reports on cytologic findings of benign PEComa all used conventional smear method.

Histologically, benign PEComas are composed mainly of sheets of polygonal cells and occasional spindle cells. Those polygonal cells have round to oval nuclei with granular chromatin and clear cytoplasm with distinct cell borders. The cells look bland and show low proliferative index. Multiple thin-walled and dilated vessels throughout the tumor are a characteristic feature. Tumor cells generally show immunoreactivity with HMB-45, Melan-A, and vimentin. They are negative for cytokeratin, TTF-1, and neuroendocrine markers. Reactivity for S-100 protein is variable.

Our case was generally similar to the typical findings of benign PEComas of the lung. However, it was different from other cases in that the thin-walled vessels were surrounded by densely hyalinized stroma.

In 2008, Hornick and Fletcher reported an analysis on sclerosing PEComa as a distinctive variant. PEComas showing more than 50% stromal hyalinization were designated as sclerosing PEComa. Among the total 70 cases collected from 1996 to 2006, only 13 cases fulfilled the criterion. Similar to conventional PEComas, those tumors were composed of bland-looking round to oval cells with clear or eosinophilic cytoplasm. The most distinguishable feature that differed from the conventional PEComa was excessive hyalinization of the stroma. On the basis of this report, we diagnosed our case as benign sclerosing PEComa.

In the collected cases, stromal hyalinization showed cord-like, trabecular or rarely, nested growth pattern. Eight out of 13 benign sclerosing PEComas arose in retroperitoneum, while remains were found in the abdominal wall or pelvis. Other reports described several cases in female genital tract and pararenal area. There has been no case depicted as benign sclerosing PEComa of the lung. In addition, no reports on cytologic findings of benign sclerosing PEComa of any organs exist to our knowledge.

When considering sclerosing variant of benign PEComa in the aspiration cytology specimen from the lung, sclerosing pneumocytoma should be included in differential diagnosis. Sclerosing pneumocytoma is usually found incidentally on chest X-ray as a single round mass. Typical cytologic findings of sclerosing pneumocytoma include blood spaces surrounded by epithelial cell-like tumor cell aggregates. Tumor cells have round to oval nuclei, finely distributed chromatin, and small nucleoli. Tumor cells may aggregate around sclerotic stromal cores. These findings might be confusing with those of benign sclerosing PEComa of the lung, which may show small blood vessels with surrounding neoplastic cells and hyalinizing material.

Differential diagnosis for typical benign PEComa of the lung includes primary clear cell adenocarcinoma of the lung and metastatic clear cell renal cell carcinoma. Primary clear cell adenocarcinoma of the lung shows clusters or single cells with ill-defined cell borders and pale cytoplasm. Nuclei are pleomorphic and have prominent nucleoli. Metastatic clear cell renal cell carcinoma shows round to polygonal cells which make loosely cohesive clusters. Tumor cells have abundant pale cytoplasm and round to oval nuclei. While nuclear atypia is mild in low-grade clear cell renal cell carcinoma, high-grade tumors show marked pleomorphism with occasional macronucleoli.

It is difficult to diagnose benign PEComa of the lung by fine needle aspiration only. If an asymptomatic patient has a coin-shaped mass in the lung periphery, cytologic features mentioned above are helpful for the differential diagnosis of other pulmonary tumors with clear cell features. Before diagnosing sclerosing variant of benign PEComa, the possibility of sclerosing pneumocytoma should always be considered.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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A Rare Case of Pulmonary Arteriovenous Hemangioma Presenting as a Peribronchial Mass

Soomin Ahn · Sejin Jung · Jong Ho Cho · Tae Sung Kim · Joungho Han
Departments of Pathology and Translational Genomics, Thoracic Surgery, and Radiology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Vascular tumors in the lung are unusual. The majority of previous reports described cases of multiple or solitary lymphangiomas or capillary and cavernous hemangiomas. To date, there has been only one report describing arteriovenous malformation/hemangioma (AVMH) in the middle mediastinum. Herein, we report an unusual case of pulmonary AVMH presenting as a peribronchial mass.

CASE REPORT

A 62-year-old man presented with a 1-month history of sputum and fatigue. He was afebrile, and routine physical examinations were within normal limits. He is an ex-smoker with a 40-pack years smoking history and denied any other medical history. Routine laboratory tests showed no abnormality. Chest computed tomography (CT) identified a 45-mm-sized mass-like lesion located centrally in the apical segment of the left upper lobe (Fig. 1A). Although the CT findings were not diagnostic, the possibility of infectious condition was favored rather than neoplasic. The patient took antibiotics for 45 days, but experienced no symptom relief, and follow-up chest CT showed no interval change of the lesion. Additional positron emission tomography was performed to characterize the lesion. A 55 × 24-mm-sized soft tissue mass showed mild heterogeneous fludeoxyglucose uptake in the lesion, suggesting malignancy (Fig. 1A). Under the clinical impression of lung cancer, left upper lobectomy was planned. Although video-assisted thoracoscopic surgery was initially attempted during the operation, it was converted to thoracotomy due to mediastinal pleural adhesion.

On the cut section of the lobectomy specimen, the dilated lumen of the lobar bronchus was packed with necrotic and mucoid material (Fig. 1B). Bronchiectasis was noted along the bronchial tree. There was no definite endobronchial lesion. Interestingly, serial cut sections of the peribronchial area revealed an ill-defined white-yellow rubbery mass-like lesion that measured approximately 2.8 cm in total extent and had pinpoint-sized spaces (Fig. 1B). Microscopically, the lesion consisted of multiple thick and prominent vessels with intervening connective tissue, and it grew between bronchial mucosa and hyaline cartilage plates (Fig. 1C). The vessels were variably sized, up to 4 mm in diameter with a thickness of up to 0.8 mm (Fig. 1D). Elastic stain visualized no definite elastic lamina, which implied the lesion to be composed of arterialized vein (Fig. 1E). These histologic findings were characteristic of AVMH. The patient was discharged without any postoperative complication. This study was approved by the Institutional Review Board of Samsung Medical Center (IRB File No. SMC 2015-07-194).

DISCUSSION

Vascular tumors of the lung are extremely rare. We presented an unusual case of pulmonary AVMH clinically mimicking lung cancer. This hemangioma presented as a peribronchial mass leading to post-obstructive bronchiectasis with mucin impaction.

The recent World Health Organization classification intro-
duces four entities of vascular neoplasm in the mediastinum: lymphangioma, hemangioma, epithelioid hemangioendothelioma, and angiosarcoma. Of the benign entities, lymphangioma is composed of medium- or small-sized lymphatic channels filled with lymphatic fluid, and hemangioma can be cavernous or capillary. To date, there have been only a few reports of multiple or solitary lymphangiomas and hemangiomas in the lung. The present tumor had histologic features of AVMH characterized by complex thick-walled vessels. To the best of our knowledge, this is the first report describing AVMH arising in the peribronchial soft tissue. Deep-seated AVMH usually arises in the limbs or head and neck. Mizutani et al. previously reported arteriovenous hemangioma in the middle mediastinum.

The literature review showed that abnormal communications between pulmonary arteries and pulmonary veins have been given various names including pulmonary arteriovenous fistulae, arteriovenous aneurysm, and AVMH. Among these, pulmonary arteriovenous fistulae refer to abnormally dilated vessels that provide a right-to-left shunt between the pulmonary artery and pulmonary vein, thereby bypassing the pulmonary capillary bed. Pulmonary arteriovenous fistulae are mostly congenital, and the majority of patients have hereditary hemorrhagic telangiectasia. These lesions have been described as pulmonary AVMH in several reports. On the other hand, AVMH is different from arteriovenous fistulae in that AVMH is an acquired tumor-like condition characterized by complex thick-walled vessels with no evidence of fistula formation. In the present case, there was no evidence of obvious fistula or shunt formation on chest CT. To avoid misunderstanding and confusion, clarification of terminology for pulmonary vascular lesion is warranted.

**Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.
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Soft Tissue Roasi-Dorfman Disease with Features of IgG4-Related Disease in a Patient with a History of Acute Myeloid Leukemia

Cheol Keun Park · Eun Kyung Kim · Ji-Ye Kim · Hayoung Woo · Mi Jang · Hyang Sook Jeong · Woo Ick Yang · Sang Kyum Kim
Department of Pathology, Yonsei University College of Medicine, Seoul, Korea

Rosai-Dorfman disease (RDD), also known as sinus histiocytosis with massive lymphadenopathy, was first described by Rosai and Dorfman in 1969. A subtype of this disease shows overlapping features with IgG4-related disease. Soft tissue involvement of RDD is very rare, and there has been no report of RDD associated with acute myeloid leukemia to date. Here, we describe a case of soft tissue RDD (STRDD) with features of IgG4-related disease in a patient with a history of acute myeloid leukemia.

CASE REPORT

Authorization for the use of the case information and materials was obtained from the Institutional Review Board of the Yonsei University College of Medicine (4-2015-0612).

A 12-year-old male patient presented with a recently developed, hard, non-tender mass in the right thigh. He had been diagnosed with acute leukemia 4 years earlier and has been in complete remission after receiving standard chemotherapy and bone marrow transplantation. Magnetic resonance imaging showed a 3.8 × 2.8 cm enhanced soft tissue mass in the subcutaneous layer on the posteromedial side of the right mid-thigh that was suggestive of leukemic infiltration (Fig. 1A). Grossly, the excised lesion showed an irregular, tan-white appearance with sclerotic areas (Fig. 1B). Microscopic examination revealed dense lymphoplasmacytic and histiocytic infiltration into sclerotic stroma involving the subcutaneous layer and deep dermis (Fig. 2A, B). Dilated sinuses were filled with large histiocytes containing intact lymphocytes (emperipolysis) (Fig. 2C, D), and these histiocytes were positive for CD68 and S100 protein (Fig. 3A, B). CD117- and CD34-positive atypical blastic cell infiltration was not identified in any of the submitted sections (data not shown). Up to 70 IgG4-positive plasma cells per high power field were noted, and the IgG4/IgG ratio was 25% in the most IgG4-positive area (Fig. 3C, D). No evidence of recurrence has been found in imaging workups during a 12-month follow-up.

DISCUSSION

RDD frequently involves extranodal sites, observed in 43% of cases. However, isolated extranodal involvement is unusual, and soft tissue involvement is even more rare. Aside from a few case reports, only a few large-scale studies have assessed STRDD. In a previous study, most STRDD presented as a rapidly growing mass and was frequently found in trunk and proximal extremities. Multicentricity was occasionally demonstrated in STRDD; however, associated lymphadenopathy was not common. Other studies have reported female predominance for STRDD over a wide range of ages with variable-sized masses. A higher recurrence rate has been suggested for STRDD with multicentric lesions compared to solitary lesions. The present case was a solitary lesion at diagnosis, and the patient is receiving regular follow-up.

Various degrees of sclerosis and IgG4-positive plasma cells have been noted in the RDD literature. A recent study reported that about 30% of RDD cases showed stromal sclerosis and increased number of IgG4-positive plasma cells, sharing similar...
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Histologic features to IgG4-related disease. These results assumed that RDD with features of IgG4-related disease could possibly be on the spectrum of IgG4-related disease or the certain phase of RDD. However, a general consensus regarding cut-off values for IgG4-positive plasma cells and the IgG4/IgG ratio has not been established for patients with RDD. The patient described here had dense lymphoplasmacytic infiltrations in a sclerotic stroma with increased number of IgG4-positive plasma cells.

The differential diagnoses showing similar histologic features
Park CK, et al. to RDD include inflammatory myofibroblastic tumor (IMT), Langerhans cell histiocytosis (LCH), and leukemic infiltration. Some IMTs show compact spindle cell proliferation with fibrosis and diffuse inflammatory cell infiltration, similar histologic features to RDD. IMTs show membranous expression of anaplastic lymphoma kinase (ALK) in immunohistochemistry. We could not find ALK reactivity in this case (data not shown) and excluded the possibility of IMT.

LCH shows histiocytic aggregation in the background of mixed inflammatory cells, a similar morphologic feature to RDD. However, we could not identify the histologic characteristics of LCH exhibiting Langerhans cells with elongated nuclei and occasional nuclear grooves positive for S100 protein and CD1a.

Considering our patient's clinical history of acute myeloid leukemia, leukemic infiltration can also be considered as a differential diagnosis. Leukemic infiltration shows infiltration of atypical blast cells in the fibrotic background, with frequent subcutaneous involvement. We assessed the possibility of leukemic involvement via immunohistochemical staining for CD117 and CD34, but did not observe immunoreactive atypical blast cells.

RDD is a rare disease entity and can have overlapping histologic features with IgG4-related disease. STRDD is even more rare and presents as a rapidly growing mass, making it difficult to be differentiated from other malignancies. In this case report, we described a case of STRDD with characteristic histologic features of IgG4-related disease in a patient with a history of acute myeloid leukemia.

Conflicts of Interest
No potential conflict of interest relevant to this article was reported.

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