PD-L1 Testing in Non-small Cell Lung Cancer
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PD-L1 Testing in Non-small Cell Lung Cancer: Past, Present, and Future

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The treatment of non-small cell lung cancer (NSCLC) has undergone a drastic paradigm shift since the introduction of immune checkpoint inhibitors (ICIs), primarily programmed cell death-1 (PD-1) and programmed death-ligand 1 (PD-L1) inhibitors. Clinical trials have demonstrated that anti–PD-1/PD-L1 agents (such as nivolumab, pembrolizumab, and atezolizumab) have remarkable anti-tumor activity and that treatment with these factors resulted in prolonged overall survival in NSCLC patients.1-6 Accordingly, these agents have been approved by the United States Food and Drug Administration (FDA) as second-line or first-line therapies for NSCLC, and clinical trials with novel agents have also shown promising results.7,8 Subsequent studies have indicated that the cell-surface expression of PD-L1 protein is an effective biomarker for predicting the response to these drugs; PD-L1 immunohistochemistry (IHC) is to date the only testing method to guide the administration of anti–PD-1/PD-L1 agents in NSCLC patients.1-6 Recently, anti–PD-1/PD-L1 agents have been integrated into the treatment option for advanced NSCLC patients. The National Comprehensive Cancer Network guidelines recommend that all advanced NSCLC samples be tested with PD-L1 IHC in a reflex manner.9

In this review, we summarize the current status of PD-L1 testing in NSCLC and discuss the major issues that can arise when applying it to clinical practice.

PROGRAMMED DEATH-LIGAND 1 PROTEIN EXPRESSION AS A BIOMARKER IN LUNG CANCER: RATIONALE AND PERFORMANCE

The immune system is regulated through a number of receptor-ligand interactions to protect the host from external antigens and prevent autoimmune reactions.10 The interaction of PD-1 expressed on cytotoxic T lymphocytes, and PD-L1 on antigen-presenting cells is one such example of an interaction (immune checkpoint).11 A tumor cell with variable neoantigens is recognized as non-self and is attacked by the immune system; however, to avoid elimination, the tumor cells may express PD-L1 protein on their surface.12 Thus, blockade of this PD-1/PD-L1 interaction by monoclonal antibodies against either PD-1 (nivolumab and pembrolizumab) or PD-L1 (atezolizumab, durvalumab, and avelumab) seems a logical therapeutic approach, especially for a highly antigenic tumor like NSCLC.13 However, the mechanisms of PD-1/PD-L1 blockade therapy and PD-L1 testing are completely different from those of EGFR, ALK, and ROS1 testing, which inhibit addictive driver oncoproteins in lung cancer. ICIs block only the interaction, which is a...
part of the normal functioning of the immune system. Therefore, the clinical effect or duration of the PD-1/PD-L1 blockade response will be different from those of receptor tyrosine kinase inhibitors. PD-L1 is a protein that is expressed with biological continuity and shows profound intra-tumoral heterogeneity, unlike genetic variation, which is separated by a binary system. It is important to choose the correct cutoff levels to define biomarker-positive and -negative patient groups for PD-L1 testing to have a predictive value. In addition, IHC for detecting protein activity may be influenced by the choice of various factors including primary antibody clones, detection system, and platforms related to complex biochemistry.

PD-L1 expression assessment is now established as a routine practice but is not without challenges. Understanding these inherent characteristics of PD-L1 testing is an important basis for pathologists to correctly interpret PD-L1 IHC results and communicate with clinicians to recommend the most effective treatment options.

**VARIOUS PROGRAMMED DEATH-LIGAND 1 ASSAY AND HARMONIZATION**

The development of ICIs was led by high-profile clinical trials, and each pharmaceutical company designed a distinct PD-L1 IHC assay to support the clinical efficacy of their own drug. Therefore, four commercial antibodies are currently available to measure PD-L1 protein expression in formalin-fixed, paraffin-embedded (FFPE) lung tissue specimens. Each assay utilizes a different automated staining system, detection system, and even means of assessment and thresholds to determine positive PD-L1 protein expression (Table 1).

However, this situation has resulted in a dilemma for pathologists. It is impractical to perform several different assays to detect one protein in a pathology laboratory with limited resources. The number of PD-L1 assays that can be performed in conjunction with the required biomarker testing (EGFR, ALK, etc.) is also limited due to the limited availability of tumor tissue, especially for small biopsy specimens from patients with advanced lung cancer. In addition, selecting one assay from the several available ones is also challenging. While each of the four IHC assays successfully recognizes PD-L1 protein, each antibody has been developed to bind to a specific epitope, and each detection system is also applied differently. Thus, the performance of these assays may be different and each assay has a cutoff to predict drug response that was determined through a clinical trial, suggesting limited interchangeability of the assays in clinical practice.

As a consequence, a number of international projects have been launched to standardize the various PD-L1 assays. The most prominent result of the Blueprint project, led by the International Association for the Study of Lung Cancer, has been relatively promising. SP263, 22C3, and 28-8 showed a high concordance in the percentage of PD-L1 membrane staining of tumor cells at any intensity; conversely, a lower expression of PD-L1 in tumor cells was observed using the SP142 clone. Following harmonization studies showed consistent results, and pathologists had hoped that several PD-L1 assays could be used interchangeably.

**Table 1. Summary of PD-L1 assays approved for non-small cell lung cancer testing**

<table>
<thead>
<tr>
<th>Performance</th>
<th>Clone</th>
<th>Developer</th>
<th>Host species</th>
<th>Epitope location</th>
<th>Platform</th>
<th>Detection kit</th>
<th>Amplification</th>
<th>Interpretation</th>
<th>Pharma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Performance</td>
<td>22C3</td>
<td>Dako</td>
<td>Mouse monoclonal</td>
<td>Extracellular domain</td>
<td>Link 48 autostainer</td>
<td>Envision FLEX</td>
<td>No</td>
<td>TC</td>
<td>Pembrolizumab</td>
</tr>
<tr>
<td>Performance</td>
<td>28-8</td>
<td>Dako</td>
<td>Rabbit monoclonal</td>
<td>Extracellular domain</td>
<td>Link 48 autostainer</td>
<td>Envision FLEX</td>
<td>No</td>
<td>TC</td>
<td>Nivolumab</td>
</tr>
<tr>
<td>Performance</td>
<td>SP263</td>
<td>Ventana</td>
<td>Rabbit monoclonal</td>
<td>Cytoplasmic domain</td>
<td>Optiview</td>
<td>No</td>
<td>TC</td>
<td>Membranous ± cytoplasmic</td>
<td>Durvalumab</td>
</tr>
<tr>
<td>Performance</td>
<td>SP142</td>
<td>Ventana</td>
<td>Rabbit monoclonal</td>
<td>Cytoplasmic domain</td>
<td>Optiview</td>
<td>No</td>
<td>Yes</td>
<td>Membranous ± cytoplasmic</td>
<td>Atezolizumab</td>
</tr>
</tbody>
</table>

PD-L1, programmed death-ligand 1; TC, tumor cell; IC, immune cell; FDA, Food and Drug Administration.

*Applied only in Korea; **Approved by Conformite Europeenne (CE) and Korea Food and Drug Administration.
for the prescription of any of PD-1/PD-L1 blockade.\textsuperscript{15-18} In addition, although 22C3 assay has been developed for use on the Dako platform, not every pathology laboratory has the Dako Autostainer, whereas the Ventana BenchMark platforms are more common in pathology laboratories. Several studies reported that the results of the 22C3 assay had shown a high correlation with those of the SP263 assay.\textsuperscript{19-22} Based on these results, the SP263 assay gained Conformite Europeanne approval for nivolumab and pembrolizumab treatment as a complementary diagnostic test.\textsuperscript{23} In Korea, the SP263 assay was also approved by the Korea FDA for nivolumab treatment. Although some studies have shown discordance between the SP263 and 22C3 assays,\textsuperscript{24,25} these discrepancies are recognized as due to the heterogeneity of PD-L1 expression or interobserver variability rather than due to the difference in assay performance. However, it is still burdensome that the misclassification of patients by using different PD-L1 assays interchangeably may lead to patients either not receiving the needed PD-1 blockade therapy, or receiving a treatment that is not beneficial. Strictly speaking, there is no gold standard assay that accurately measures PD-L1 expression and best predicts PD-1 blockade response.

**INTERPRETATION AND PATHOLOGICAL REPORTING OF PROGRAMMED DEATH-LIGAND 1 IMMUNOHISTOCHEMISTRY**

For the reasons mentioned above, several PD-L1 assays are generally performed simultaneously or sequentially in many pathology laboratories for the prescription of anti–PD-1/PD-L1 agents. The interpretation of PD-L1 IHC assays is a challenge for pathologists because of the different methods of interpreting positive results and in the different cutoff values for each assay (Table 1).

For the definition of positive PD-L1 staining, complete circumferential or partial linear membranous staining of tumor cells at any intensity is considered positive for the 22C3 and 28-8 assays, while any membranous and/or cytoplasmic expression of tumor cells is considered positive for the SP263 and SP142 assays (Fig. 1). In the SP142 assay, PD-L1–positive immune cells, as well as tumor cells, are considered in the criteria for positive PD-L1 staining. Interpretation issues relating to distinguishing tumor cell PD-L1 chromogenic signals from those of inflammatory cells, the mislocalized signal from the membrane to the cytoplasm (for 22C3 and 28-8), and the scoring of percentages of expression particularly around the thresholds of clinical significance are always a concern for pathologists, regardless of the assay type. Furthermore, each assay has a specific cutoff value for positive tumor cells, and the percentage may be different depending on whether it is the first line vs. second or further line of treatment, even for the same drug. In addition, when an assay is applied for two drug prescriptions, such as SP263, different cutoffs may be applied depending on the drug. The SP263 assay uses two different cutoffs; 25% for durvalumab and 10% cutoff for nivolumab, in Korea (Table 1). Because these inconsistent cutoffs are not proven by clinical trial, but a special situation in Korea related to national insurance, pathologists also have to pay close attention to the insurance policies related with PD-L1 testing.

Given these differences, pathological reports on PD-L1 IHC should be more comprehensive, giving more than a simple positive or negative result. The name of the diagnostic kit used, the assessment score, information on the required minimum number of cells that were assessed, and any comment on the meaning of the score with regard to the cutoff of that specific test should be included in the report. If the immunotherapeutic agent to be used is known at the time of testing, the results can be reported in terms of broader categories (e.g., \(< 1\%\), 1\%–49\%, and \(> 50\%\) with 22C3 for pembrolizumab treatment), appropriate for recom-

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**Fig. 1.** Representative images stained with the programmed death-ligand 1 immunohistochemical assays: 22C3 (A), SP263 (B), and SP142 (C).
mending treatment with the drug of interest. The interpretation and reporting for PD-L1 IHC assays in lung cancer samples differs from those for conventional IHC in that a wide variety of assays need to be interpreted according to the relevant criteria for each drug. Therefore, specialized training is important to maintain the consistency and quality of interpretation between pathologists.

**ONGOING ISSUES**

**Use of archival tissue or need for re-biopsy**

Concerns about performing PD-L1 testing using archival samples may be due to two reasons: (1) it is unclear whether antigenicity is preserved in the archival samples, and (2) treatment may alter the patterns of PD-L1 protein expression. The answer to the first concern seems to have been resolved by a recently updated analysis of the KEYNOTE-010 trial. They showed the overall survival benefit of pembrolizumab over docetaxel for both TPS ≥ 50% and ≥ 1%, regardless of whether PD-L1 was assessed in 456 archival or 578 newly collected tumor samples. Compared with newly collected tumor samples, archival samples were not associated with a loss of PD-L1 expression, suggesting that both newly acquired biopsy samples and aged archival specimens are suitable for PD-L1 testing.

Regarding the second issue, several reports have explored the changes in PD-L1 expression during the clinical course of NSCLC patients in relation to locoregional and/or systemic treatment. Omori et al. demonstrated that major changes in PD-L1 expression were observed in 38% of a total of 76 NSCLC patients undergoing anticancer treatments, including systemic chemotherapy or targeted therapy, as well as only surgical resection. The effect of anticancer drugs on the expression of PD-L1 may be affected by the differences between agents as well as the characteristics of the tumor that affect the mechanisms of host immune system modulation. Recent results from several studies investigating the alterations in tumor PD-L1 expression in patients with NSCLC who received platinum-based neoadjuvant chemotherapy produced conflicting results, proving the importance of considering tumor-specific characteristics. It has been reported that PD-L1 expression is increased by EGFR signaling conferred by the activation of EGFR mutations and that erlotinib could downregulate PD-L1 expression. Conversely, several reports showed EGFR-TKI treatment appeared to increase PD-L1 expression in tumor cells with EGFR mutations. Although the mechanism of these changes has not been elucidated, these discrepancies may be explained by differences in patient characteristics, such as tumor stage and anticancer treatments other than EGFR-TKI, which could lead to differences in PD-L1 expression. These dynamic properties of PD-L1 expression provide a possible explanation for the second- or further line treatment responses of PD-1/PD-L1 blockade therapies in treatment-naive samples with negative status. However, there is a lack of clinical data on the degree of accuracy with which the altered levels of PD-L1 expression after treatment predicts the response to immunotherapeutic agents. It is important to evaluate PD-L1 expression in serial samples throughout the treatment, and at least in the latest tumor specimen, especially for heavily treated NSCLC patients.

**Heterogeneity**

PD-L1 expression may show intratumoral or intertumoral heterogeneity; therefore, it is important to understand that the sampling method (surgical resection vs biopsy) and sites (primary vs. metastasis) may influence the PD-L1 expression status. Several studies reported inconsistencies in the PD-L1 status of resected versus biopsied specimens. A comparison of PD-L1 expression by Ilie et al. using an SP142 clone between preoperative biopsy specimens and their corresponding resected specimens in 160 NSCLC patients found a significant discordance (overall discordance rate = 48%; κ value = 0.218). The authors noted that most (75%) discordant cases were based on the assessment of PD-L1 staining in immune cells. Gniadek et al. compared four tissue microarray cores from 150 FFPE tissues of resected primary cancers using the SP142 clone. They found substantial inconsistencies in the percentages of PD-L1–positive cells in different tissue microarray cores in both 71 adenocarcinomas and 79 squamous cell carcinomas. In our previous study, we used the 22C3 assay for comparison, as 22C3 showed the highest tumor proportion score and may reduce the effects of intratumoral heterogeneity in PD-L1 expression. However, seven of the 50 cases (14%) exhibited discordant PD-L1 expression between the tissue microarray cores and resected specimens.

Differences in PD-L1 expression between primary and metastatic lesions appear to be less important than the differences in sampling methods. Mansfield et al. assessed the PD-L1 expression in 67 paired, resected multifocal lung cancers from thirty-two patients. They observed a strong consistency in PD-L1 expression in tumor cells among related, metastatic multifocal lung cancers; conversely, there was a low correlation of PD-L1 expression between multiple independent lesions. Kim et al. evaluated PD-L1 expression in 161 paired primary and metastatic adenocarcinoma tissues from 146 lung cancer patients using an E1L3N clone. Their study demonstrated that the concordance

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Additional studies have reported high conformity of PD-L1 expression between primary and metastatic tumors was 80.1% \( (k = 0.492) \) and 90.7% \( (k = 0.598) \) with a 1% cutoff, respectively.\(^{17}\)

This heterogeneity of PD-L1 expression is a major obstacle for PD-L1 testing; it may not be a perfect predictive biomarker for PD-1/PD-L1 blockade treatment, and this could be one of the reasons for the suboptimal correlation between PD-L1 expression and treatment responses. However, it is not practical to perform multiple biopsies at one or multiple sites to assess PD-L1 expression. Recently, novel techniques for testing PD-L1 expression using imaging\(^{38}\) or peripheral blood\(^{39,40}\) have been examined.

**Cytological specimens**

Currently, PD-L1 IHC is applicable to histologic samples only and is not recommended in cytologic samples, because cytologic materials were excluded for PD-L1 assessment in clinical trials. However, about one-third of patients with metastasis are still diagnosed by cytological materials only, which often is the only sample that can be used for PD-L1 testing. This has made some pathologists curious about the clinical use of cytology samples for PD-L1 testing.

Rebelatto et al.\(^{41}\) showed that 95% alcohol, AFA, and Prefer are unsuitable fixatives for IHC with the SP263 clone. Evaluation of PD-L1–positive immune cells using the Ventana SP142 assay will likely be more challenging in cytological specimens, as the lack of tissue architecture precludes the ability to distinguish immune cells within the tumor area from those outside tumor boundaries that are considered irrelevant for PD-L1 scoring.

Using DAKO 28-8 and 22C3 clones, Skov and Skov\(^{42}\) compared PD-L1–expression levels in 86 paired FFPE samples of cytologic cell blocks and histological materials from lung malignancies and observed a high degree of consistency between histologic and cytologic specimens within each assay. In cases showing discrepancies between the two sample types, the tumor tended to demonstrate heterogeneous PD-L1 staining in the histologic material, especially for PD-L1 expression ≥ 5% and ≥ 10%.\(^{42}\)

Additional studies have reported high conformity of PD-L1 expression between cell blocks and matched histological specimens and/or comparable PD-L1 expression among cell blocks, small biopsies, and resections in a prospective cohort using the 22C3 clone.\(^{43,44}\) These data suggested that the assessment of PD-L1 expression in tumor cells can also be performed using cytologic materials that are processed to obtain cell blocks, and could be an alternative when histological samples are not available, at least when PD-L1 expression is detected.

However, before recommending the routine clinical use of cytologic specimens, a standardized process should be established to account for the wide range of processing methods, including cell collection (e.g., aspiration, liquid-based, and cell block) and fixation (e.g., alcohol-based and formalin). Further large-scale validation studies are warranted to establish standardized PD-L1 IHC testing methods for cytology specimens.

**BEYOND PROGRAMMED DEATH-LIGAND 1: IS THERE ANY PREDICTIVE BIOMARKER AS AN ALTERNATIVE TO PROGRAMMED DEATH-LIGAND 1?**

PD-L1 IHC is the sole biomarker currently available for analysis; unfortunately, it is not an optimal biomarker owing to several major limitations, as discussed above. At present, there is a need to discover and validate additional predictive biomarkers other than PD-L1 IHC to improve patient selection and spare unnecessary toxicity and costs in non-responders. Various additional factors are under investigation, including the tumor mutation burden (TMB),\(^{45,46}\) tumor-infiltrating lymphocytes,\(^{47,48}\) and immune gene signatures\(^{49,50}\) that may identify tumors with preexisting immune activity and be correlated with the response to anti–PD-L1/PD-1 agents. Peripheral circulating immune cells and T-cell receptor diversity may be reflective of the tumor microenvironment, though this has yet to be validated in clinical practice.\(^{49,50}\) Finally, although the gut microbiome is showing exciting promise as a marker for immune-checkpoint efficacy, its predictive value needs to be validated in larger clinical studies.\(^{51}\)

Of these, TMB defined as the total number of non-synonymous somatic mutations in the tumor genome is emerging as a predictive biomarker of response to ICIs in various cancers including NSCLC. Non-synonymous somatic mutations alter the amino acid sequence of proteins encoded by affected gene, forming neoantigens. It is hypothesized that neoantigen formation contributes to the intrinsic immunogenicity of a tumor.\(^{45,52}\) In support of this premise, a higher TMB has also been shown to correlate with clinical benefit from ICI therapy in NSCLC,\(^{45,46}\) as well as small cell lung cancer,\(^{53}\) melanoma,\(^{52}\) and colorectal cancer.\(^{54}\)

While whole exome sequencing (WES) is widely considered the gold standard for measurement of TMB,\(^{45,55}\) performance of WES is currently impractical for several reasons including cost and turnaround time. Targeted panel sequencing has offered a practical estimate of TMB from the whole exome in the clinical setting.\(^{46,56}\) Because the thresholds that define high TMB level vary, and reported values also depend on the different techniques used, it is important to harmonize and standardize TMB assay
methods and reporting to ensure the successful implementation of clinical TMB testing. Ongoing efforts to ensure reproducible assessment and reporting standards will facilitate the smooth implementation of TMB testing for cancer immunotherapy.

CONCLUSION

Much remains uncertain about the clinical response to PD-1/PD-L1 blockade therapy in NSCLC; however, it is very clear that one single test cannot be used as a reproducible surrogate to predict the benefit of immunotherapy. Rather, reflecting the clinical complexity of combination multi-modality therapies, the development of a predictive model that takes into account the complex components that affect tumor-host interactions is needed. Although pathologists need to face the practical reality that oncologists will regularly request the PD-L1 IHC results, it should also be considered that there may be room for improvement in terms of the biomarkers for immunotherapy response, and that PD-L1 expression alone is often insufficient for patient stratification for PD-1/PD-L1 blockade therapy.

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Methodology: HK, JHC.
Supervision: JHC.
Writing—original draft: HK.
Writing—review & editing: HK, JHC.

Conflicts of Interest

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

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How to Foster Professional Values during Pathology Residency

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The importance of professional and ethical behavior by physicians both in training and in practice cannot be overemphasized, particularly in pathology. Professionalism education begins in medical school, and professional attitudes and behaviors are further internalized during residency. Learning how to be a professional is a vital part of residency training. While hospital- or institution-based lecture style educational programs exist, they are often ineffective because the curriculum is not applicable to all specialties, although the basic concepts are the same. In this paper, the author suggests ways for institutions to develop professional attitude assessments and to survey residents’ responses to various unprofessional situations using case scenarios.

Key Words: Professionalism; Pathology residency; Case-based education; Assessment

The recognition and importance of medical ethics and professionalism in the practice of medicine has substantially increased. As a result, bioethics and medical humanity education have become hot topics in Korean medical education programs. However, these programs’ ineffectiveness is due to the inadequate number of experts, many of whom fail to set definite and detailed educational goals. In addition, there is a lack of a general consensus on the need for and purpose of professionalism education in Korea. Medical schools focus on theory-based education, which hinders medical students in developing a sense of empathy or desperation. Moreover, there are currently no appropriate education or evaluation programs to train pathology residents in the ability to apply medical ethics and professionalism. The current curriculum in medical school focuses instead on the professionalism and medical ethics of physicians rather than pathologists. Therefore, the author suggests an exclusive definition of professionalism in pathology and ways to foster such a concept in the course.

DEFINITION OF PROFESSIONALISM

Definitions of professionalism vary across different organizations and fields. Webster’s Dictionary defines professionalism as “a calling requiring specialized knowledge and often long and intensive preparation including instruction in skills and methods as well as in the scientific, historical, or scholarly principles underlying such skills and methods; maintaining by force of organization or concerted opinion high standards of achievement and conduct, and committing its members to continued study and to a kind of work which has for its prime purpose the rendering of a public service.” The American Board of Internal Medicine states that “professionalism in medicine requires the physician to serve the interests of the patient above his or her self-interest. Professionalism aspires to altruism, accountability, excellence, duty, service, honor, integrity, and respect for others.” The Accreditation Council for Graduate Medical Education of the United States has recently mandated that all residency training programs adopt and incorporate competency-based residency training in six defined areas. One of these is professionalism where, among other requirements, residents are expected “to demonstrate a commitment to carrying out professional responsibilities and adherence to ethical principles.” Although the Council expects each training program to develop its own unique approach to incorporating and measuring outcomes in the general competencies, there is little information on how to assess, measure, and teach professionalism, including bioethics. Therefore, much can be gained by sharing information within and across specialty areas, especially in the pathology field.
PROFESSIONALISM IN PATHOLOGY

As a medical specialty, pathology deals with the interpretation of changes in human tissues and cells that cause or are caused by disease. In addition to making these diagnoses, pathologists also determine the causes and effects of death and diseases through autopsy and molecular pathology techniques. Pathologists are thus laboratory-based physicians. Issues of medical ethics and professionalism might seem a distant consideration because pathologists do not usually come in direct contact with patients in their daily practice or even during their pathology residency training. One study found that ethical and professionalism issues, such as honesty, recognizing, and reporting medical errors, interpersonal interactions, and conflicts of interest, were some of the most important issues in the pathology profession. While that study involved pathologists already in practice, these concerns and behaviors are just as important for those in training who will soon enter practice. Other professionalism issues that can manifest during residency training include a negative attitude, interpersonal conflicts, or other inappropriate behavior toward staff, peers, faculty, or patients.

How to teach or foster professionalism during a pathology residency

First, the concept of professionalism should be added to the “competence of residency” dimension of the educational goals of the Korean Society of Pathology (KSP). The current description of a successful residency states: “Through four years of training courses, residents should acquire the basic concepts of pathology and learn basic knowledge and skills as a pathologist. Residents should handle biopsy as well as cytology specimens by themselves and report accurate and critical diagnoses based on their pathologic findings. In addition, residents should further advance knowledge in at least one concentration during their educational course. Residents should have good communication skills to effectively seek consultations from other physicians regarding the diagnosis and treatment of patients. Residents also have to grow basic yet comprehensive skills to run a laboratory effectively.” We therefore set out to develop KSP educational content for professionalism suitable for pathologists with an emphasis on altruism, accountability, excellence, duty, service, honor, integrity, and respect for others.

The regular evaluation of a resident’s knowledge, skills, and attitude in this domain of professionalism should be a mandatory part of training. As a result, the resident’s knowledge, attitudes, and skills in this area should show an appropriate evolution during his or her training, with the mastery of more advanced attitudes and skills in being a professional appearing as the resident’s clinical training progresses. Above all, pathology faculty should try to become mentors or role models to residents.

This paper suggests two practical methods that can be easily applied by the KSP (Fig. 1). One is to make a structured daily assessment in individual departments. The second is for the KSP to modify the case-based evaluation method reported by Domen et al. to conduct a nationwide survey with the goal of self-reflection. Cases should be made by pathologists and reflect current teaching circumstances. The following are assessment forms used at McGill University that can be modified to suit each institute. The author would like to suggest creating a similar evaluation form that can be assessed by education program directors after a trial period.

Professionalism evaluation items on in-training assessment forms

Integrity and honesty

Judges whether the trainee is dependable, reliable, honest, and forthright in all information and facts.
Sensitivity and respect for diversity

Assesses whether the trainee is able to understand and be sensitive to issues related to age, gender, culture, and ethnicity.

Self-disciplined responsibility

Determines whether the trainee adequately accepts professional responsibilities, places the needs of the patients before the trainee’s own, and ensures that the trainee or his/her replacement are at all times available to the patient. The trainee is punctual and respects local regulations related to the performance of his/her duties.

Communication with other clinicians

Judges that the trainee maintains a focus on patient care with compassion and empathy.

Recognition of one’s own limitations, seeking advice when needed

Decides whether the trainee is able to self-assess his/her limits of competence and is able to seek and give assistance when necessary.

Understanding the principles of ethics as applied to clinical situations

Assesses the trainees’ understanding of the principles and practice of biomedical ethics as it relates to the specialty. The trainee practices in an ethically responsible manner.

Case-based evaluation

Domen et al.6 developed five case scenarios highlighting various unprofessional behaviors. A standard set of responses was offered to the participants; polling results were collected electronically, and the results were compared. The cases involved poor attendance, lack of attention, an alcohol odor on the breath, medication effects, dishonesty, poor interpersonal skills, unprofessional use of social media, illegal access to electronic medical records, etc. A level of generational differences appeared to be evident in this study regarding the recognition and management of unprofessional behavior; there was also agreement in some cases. These differences regarding professionalism for both faculty and residents should be considered an integral part of any educational and management approach to professionalism. Presenting or discussing appropriate actions for each scenario provides students with opportunities to recognize a variety of perspectives. The author suggests designing similar local scenarios based on a comparative analysis of residents and faculty to develop supplementary content for professionalism training.

CONCLUSION

The importance of professionalism in the practice of medicine has increased and become a vital part of residency training. While hospital based lecture style educational programs exist, they are often ineffective because the curriculum is not applicable to pathology residency, although the basic concepts are the same. In this paper, the author suggests two practical methods to foster professional values during pathology residency. One is to make a structured daily assessment in individual departments. The second is to conduct a nationwide case-based evaluation by KSP.

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Conflicts of Interest

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CURRENT STATUS OF AND PERSPECTIVES ON CERVICAL CANCER SCREENING IN KOREA

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HISTORY OF CERVICAL CANCER SCREENING

According to Globocan 2017 (International Agency for Research on Cancer [IARC]), the worldwide age-specific incidence rate of cervical cancer has been persistently decreasing, likely due to Papanicolaou (Pap) screening.1 Screening tests for cervical cancer have a long history, as cervical cancer, unlike other cancer types, is characterized by a long (7–20 years) precancerous stage prior to progression into an invasive cancer and easy detection is possible due to convenient existing diagnostic methods. Since the Pap smear system was first used by Dr. Papanicolaou in 1943 to examine cervicovaginal smears, it has been utilized as a screening tool for cervical cancer including low cost, simple procedures to obtain and manage specimens, and high specificity. The use of the Pap smear has contributed to reductions of the incidence and mortality of cervical cancer worldwide. In Korea, regular health check-ups for industrial workers and their family members were introduced in 1988 and were performed as part of the National Cancer Screening Program in 1999. As a result, the incidence of cervical cancer in Korea has been steadily decreasing. However, about 800 cases of cervical cancer-related deaths are reported each year due to false-negative test results. Hence, new screening methods have been proposed. Liquid-based cytology (LBC) was introduced in 1996 to overcome the limitations of conventional Pap smears. Since then, other LBC methods have been developed and utilized, including the human papillomavirus test—a method with higher sensitivity that requires fewer screenings. In this study, we review current issues and future perspectives related to cervical cancer screening in Korea.

Key Words: Uterus; Cervix uteri; Neoplasms; Screening; Papanicolaou test; Human papillomavirus DNA tests; Perspectives

Since the introduction of the Papanicolaou (Pap) smear system in 1943, cervicovaginal cytology has been used as a standard screening test for cervical cancer. The dissemination of this test contributed to reductions of the incidence and mortality of cervical cancer worldwide. In Korea, regular health check-ups for industrial workers and their family members were introduced in 1988 and were performed as part of the National Cancer Screening Program in 1999. As a result, the incidence of cervical cancer in Korea has been steadily decreasing. However, about 800 cases of cervical cancer-related deaths are reported each year due to false-negative test results. Hence, new screening methods have been proposed. Liquid-based cytology (LBC) was introduced in 1996 to overcome the limitations of conventional Pap smears. Since then, other LBC methods have been developed and utilized, including the human papillomavirus test—a method with higher sensitivity that requires fewer screenings. In this study, we review current issues and future perspectives related to cervical cancer screening in Korea.

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female population, as part of the bi-annual regular health checkup. The outcomes of early screening programs for cervical cancer were reported to The Bethesda System (2001). However, this did not include information regarding the duration of screening or age groups in which the test should be performed.7,6

The incidence of cervical cancer in the Korean population has been steadily decreasing, from 4,443 cases in 1999 (18.9 per 100,000 females, the third most common cancer affecting women) to 3,582 cases in 2015 (14.1 per 100,000 females, the seventh most common cancer affecting women).7 The Quality Improvement Committee of the Korean Society for Cytopathology (KSC) conducted nationwide quality control surveys in cytopathology in 1998, 2001, and 2016. Comparisons of the number of cervicovaginal cytology exams performed at medical centers (including university hospitals) and commercial laboratories participating in the survey showed that the majority of tests were performed in commercial laboratories (66.4% in 1998, 68.6% in 2001, and 74.9% in 2016). The rate of cancer diagnosis from cervicovaginal cytology has remarkably decreased, from 0.1%–0.96% at university hospitals and 0.07%–0.09% at commercial laboratories in 1998 to 0.28% at university hospitals and 0.035% at commercial laboratories in 2016.8,9 However, as about 800 cases of cervical cancer-related deaths are reported each year, concerns regarding limitations of current screening tests have been raised.7

Despite the numerous advantages of CS, this technique requires supplementation due to the high false-negative rate (20%) caused by errors occurring during specimen collection, preservation, slide generation, analysis, and readout; low sensitivity; highly subjective results, and low reproducibility; and the level of expertise of the screener or pathologist. Hence, an improved screening tool is required.10,11

In Korea, about 60% of false-negative cases based on CS arise from specimen collection.7 To overcome these limitations, a liquid-based cytology (LBC) method involving fluid-based collection and processing was developed. Since the initial introduction of the ThinPrep Pap test (Cytyc Corp., Boxborough, MA, USA) in 1996, various LBC methods have been developed to improve the Pap test. In Korea, LBC-based cervicovaginal cytology has been covered by health insurance since 2006.

LBC is thought to have the following advantages: low levels of drying artifacts, as each specimen is fixed immediately after collection; superior morphology; reduction in the number of cases with background obscuring caused by blood or inflammatory cells; quick and easy screening; better sampling due to the dispersion of cells; and the potential for multiple testing from a single sample. Previous clinical trials have demonstrated the effectiveness of LBC in increasing the rate of squamous intraepithelial lesion (SIL) detection, especially low-grade SIL (LSIL), and improving specimen adequacy.12,13

Several studies have compared the clinical utility of LBC-based cervicovaginal examination and outcomes using the CS method. The LBC method not only improved the diagnostic rate of atypical squamous cells of undetermined significance (ASCUS) and SIL, but also reduced the proportion of unsatisfactory samples, compared with the CS method.14,16

According to the results of the 2016 KSC survey, the most commonly used LBC Pap tests in Korea were as follows: ThinPrep (39.7%), Cell Prep (26.3%), SurePath (23.7%), and other (< 5%).7

A nationwide study conducted in Korea demonstrated that the number of LBCs performed during cervicovaginal cytology has steadily increased since the introduction of LBC, from 7.6% (2004)4 to 20.5% (2007)4 to 25.3% (2015). The diagnostic accuracy of the LBC method was more commonly used in commercial laboratories (CS, 79.4%; LBC, 20.6%) and general hospitals (CS, 80.9%; LBC, 19.1%), while the LBC method was more often used in university hospitals (CS, 44.1%; LBC, 55.9%).8

Unsatisfactory samples in cervicovaginal cytology are usually re-examined within a period of 2–4 months, since sampling adequacy allows for appropriate patient follow-up.17 Appropriate comparisons could not be made, since there is a limited number of Korean studies assessing unsatisfactory samples. However, according to the 2016 KSC survey, unsatisfactory samples accounted for 0.6% of all cervicovaginal cytology cases.9 As LBC can reduce the proportion of unsatisfactory samples, the proportion of unsatisfactory samples will likely decrease from 0.6% in the future.18

Moreover, diagnostic accuracy assessed by comparing cytologic cases with matching histological specimens is crucial for evaluations of false positives/negatives. The diagnostic accuracy values reported in the 2016 KSC survey were as follows: for university hospitals, category A, 9.1%; category B, 4.0%; category C, 0.6%; and category O, 86.3%; and for general hospitals, category A, 17.4%; category B, 10.2%; category C, 1.9%; and category O, 69.5%. Accuracy data from commercial laboratories were not available.9

Since Pap tests alone cannot produce accurate outcomes for cervical cancer screening, an adjunctive, combined test is required.19 The combination of Pap test and colposcopy is reported to increase sensitivity, but the limitations of colposcopy (cost, time, and expert training) make it impractical for use as a screening method.20,21 Similar to colposcopy, cervicography is another
potential adjunctive test to complement Pap tests, allowing for interpretations of ectocervical photographic images. Evaluations by cervicography are based on the principles of colposcopy.\textsuperscript{21,22}

Higher diagnostic accuracy can be achieved by utilizing the Pap test and cervicography as a combined screening method: 90\%–100\% for infiltrating cancers and 90\%–95\% for cervical intraepithelial neoplasia (CIN). However, its medical cost, despite being less than a combined method using the Pap test and human papillomavirus (HPV) test, is still a significant limiting factor for it to be used as a general screening method.\textsuperscript{23}

HPV plays an important role in the development of cervical cancer and is found in 90\%–100\% of high-grade SIL (HSIL) or invasive carcinomas. Therefore, including HPV tests in cervical cancer screening is useful for complementing traditional cytologic examinations.\textsuperscript{24} More specifically, for the epidemiological management of cervical cancer, screening of patients diagnosed with ASCUS is a clinically important issue. Repeat cervicovaginal smear tests, colposcopy, and HPV DNA tests are recommended methods to select high-risk patients diagnosed with ASCUS, and the utility of HPV DNA test is steadily emphasized.

There are two major HPV examination methods: the first involves amplifying HPV DNA by polymerase chain reaction (PCR), while the second involves direct confirmation of HPV DNA through DNA hybridization. In the 1994 Interim Guidelines, HPV tests were suggested as an adjunctive method for the management of ASCUS and LSIL.\textsuperscript{25} However, the reliability of HPV tests has been questioned due to the diverse sensitivity of this examination method. The sensitivity and reliability of the Hybrid Capture II system (Digene Corporation, Qiagen N.V., Venlo, Netherlands) have been approved by the Food and Drug Administration (FDA), and it has been widely used to test for HPV. An HPV DNA microchip test that allows for the identification of HPV genotypes from a single examination is also being utilized.

Although the majority of ASCUS or LSIL are naturally eliminated, a small portion persist or develop into more severe CIN. Furthermore, among patients initially diagnosed with ASCUS or LSIL by the Pap test, 5\%–15\% are found to have HSIL through biopsy,\textsuperscript{26} suggesting that it is important to check for HSIL in patients diagnosed with ASCUS or LSIL by the Pap test.

According to a previous study,\textsuperscript{27} HPV tests had a greater sensitivity for detecting HSIL from ASCUS than Pap tests (0.83 vs 0.66), but combination of the HPV test and Pap test has increased sensitivity (0.92). The Pap test (1.00) has higher sensitivity for detecting HSIL from LSIL compared with the HPV test (0.93), while a combination of HPV and Pap tests also showed a sensitivity of 1.00. Moreover, the ASCUS-LSIL Triage Study (ALTS) determined that HPV tests are useful for the management of ASCUS patients but not useful for the management of LSIL patients; thus, LSIL patients should undergo immediate colposcopy or Pap tests every 6 months.\textsuperscript{28,29}

A joint study performed by a Korean group and IARC selected a random region in the city of Busan to evaluate the prevalence of HPV infections via cervicovaginal cytology (PCR-enzyme immunoassay method). This study showed that 10.3\% of participants were positive for HPV DNA, while 60\% had high-risk HPV (HR-HPV). The HR-HPV types observed among infected individuals in order of frequency were HPV 16, 33, 58, 66, 18, 31, and other, while the low-risk HPV (LR-HPV) types observed in order of frequency were HPV 70, 81, and other.\textsuperscript{30}

Unlike studies of the general population, one study that selected a specific cohort of patients from the Department of Obstetrics and Gynecology at a university hospital in Korea and evaluated the prevalence of HPV infections via cervicovaginal cytology (HPV DNA microchip test). Interestingly, 48.8\% of the participants were positive for HPV DNA, while 86.9\% were positive for HR-HPV. The HR-HPV types detected among infected individuals in order of frequency were HPV 16, 58, 18, 52, 53, 31, and other, while the LR-HPV types detected in order of frequency were HPV 70, 6, 11, 40, and 42.\textsuperscript{31} Therefore, there were differences between the general population cohort and a cohort of patients from the Department of Obstetrics and Gynecology in HR-HPV and LR-HPV types.

**CURRENT STATUS OF CERVICAL CANCER SCREENING**

Cervical cancer screening tests using cervicovaginal cytology were not widely used in the early stages of screening programs. According to a 1998 survey performed by the Ministry of Health and Welfare in Korea, only 33.88\% of women (≥ 20 years old) underwent screening tests, likely due to the lack of promotion and follow-up for lower-income brackets.\textsuperscript{7} Since then, promotion among the general population and the provision of free screening tests for cervical cancer have steadily increased the rate of eligible women undergoing screening.

Cervical cancer screening is covered by international guidelines provided by the World Health Organization and the European Commission. A few European countries, including France, have established new screening programs for 2014–2019, based on pilot experiments performed in different regions of participating countries.\textsuperscript{32} Such programs will initially be based on the
Pap test, but with the objective of progressing toward direct screening for HPV infection.

A German study that assessed costs and clinical effectiveness showed that HR-HPV (HPV 16/18) tests performed in conjunction with p16/Ki-67 dual-stained cytology can improve the detection rate of cervical cancer compared to the Pap test alone, with lower total annual cost. In addition, an Australian study demonstrated that HPV tests performed every 5 years are more effective for cervical cancer screening and cost-savings than Pap tests performed every 2 years. This Australian National Cervical Screening Program switched to primary HPV screening with partial genotyping as of 2017.

Even in the United States, where the use of combined Pap plus HPV test is recommended, additional studies have been performed to determine which screening method can best improve clinical and economic outcomes, since the approval of the HPV test in 2014 as a primary cervical cancer screening tool for women aged ≥25 years. The outcomes of related studies have reported that primary HR-HPV screening performed every 5 years is the most efficient alternative screening tool to the Pap test.

Most updated guidelines for cervical cancer screening in Korea recommend that all asymptomatic women aged over 20 years should start cervical cancer screening by Pap smear (CS or LBC) and continue every 3 years until they reach the age of 74 if three consecutive cytologic examinations have been negative within the previous 10 years. These fundamental differences in strategies of cervical cancer screening between Korea and other countries (US, Australia, and European countries) are likely due to insufficient screening or poor accessibility to medical services in other countries. As a consequence, Pap tests are underused in underserved populations, and there are inequalities in access to the Pap test. The cost of HPV testing is lower than the costs associated with the establishment of cytological screening systems. In addition, noncompliant populations and individuals may perform self-sampling as they cannot meet medical staff frequently. Hence, HPV tests are recommended screening methods in low-access countries.

Although HPV testing is increasingly used for primary screening in some countries, its efficacy and cost-effectiveness vary in different clinical and social-economical settings. The Pap test is extremely useful in countries with well-developed screening systems or in populous countries like Korea. In Japan, women between 20 and 69 years of age undergo cytologic screening every 2 years. In Hong Kong, women more than 25 years of age undergo cytologic screening at one- to three-year intervals, and cervical cytology remains the most effective screening tool for population-based cervical cancer screening. Taiwan also provides cytologic screening for women more than 30 years of age at 3-year intervals.

Due to limited financial support from the national cancer screening program and the low associated medical costs when performed in obstetrics and gynecology clinics, the Pap test is still often utilized in Korea. The Pap test is considered the core of cervical cancer screening, due to the following factors: nationwide quality assurance management of Pap tests via the Korean Society for Cytopathology, available training for qualified cytology screening personnel, low accuracy of the HPV test, low reproducibility between HPV tests, and relatively high cost associated with the HPV test. Furthermore, even in HPV-negative cases, lesions equal to or more severe than mild dysplasia are observed under cytological and histopathological examinations in 17.5% of cases. Finally, despite the high sensitivity of the HPV test, it has the crucial disadvantage of low specificity and its standalone outcome is not useful in most cases, which may cause unnecessary anxiety for patients. Therefore, in the 2015 guidelines for cervical cancer screening provided by the Ministry of Health and Welfare in Korea, the HPV test is not considered an adequate standalone screening test.

**FUTURE PERSPECTIVES ON CERVICAL CANCER SCREENING IN KOREA**

Korea has socioeconomic, geographical, and ethnic differences from other countries (Australia, North American, and European countries), where the HPV test is used as a primary cervical cancer screening tool. Therefore, actual conditions in Korea must be considered prior to the establishment of new cervical cancer screening methods.

There are no systematic studies regarding the reproducibility and accuracy of HPV tests used in Korea. The uncontrolled performance of HPV tests in Korea therefore might result in increased medical costs and patient anxiety. To determine whether HPV tests should be used as primary cervical cancer screening methods in Korea, a nationwide quality assurance management protocol for HPV testing should be established, and study outcomes regarding the accuracy and relative sensitivity of HPV tests should be made available to the public. Consequently, institutions performing HPV tests should choose a validated, acceptable screening method, and quality control of screening methods must be ensured prior to the utilization of HPV tests. In addition, the health insurance costs of HPV tests are extremely high (53,480 won) compared to the Pap test (7,750 won), which is an
important obstacle to the establishment of a nationwide cervical cancer screening program.

A recently published meta-analysis confirmed the diagnostic accuracy of cytology as a primary screening test, which has higher sensitivity and specificity for the detection of SIL and squamous cell carcinoma. Therefore, cervicovaginal cytology is one of the most useful, sensitive, and confirmative primary cervical cancer screening tests available.

The revised guidelines for cervical cancer screening in Korea (2015) can be summarized as follows: “The committee recommends screening for cervical cancer in women older than 20 years of age with cytology (Pap test or LBC) every 3 years (recommendation A). The combination test (cytology with HPV test) is recommended as an option in consideration of individual risks or preferences (recommendation C). The current evidence for primary HPV screening is insufficient to assess the benefits and harm of cervical cancer screening (recommendation I). Cervical cancer screening can be terminated at 74 years old if it has been confirmed that the patient has more than 3 consecutive negative cytology results within 10 years.” As described above, HPV tests in Korea are considered useful co-tests when performed alongside the Pap test, rather than as a primary cervical cancer screening method.

Aside from the cervical cancer screening methods mentioned above, HPV vaccines, which were approved by the FDA in 2006 and have been included as a part of a cost-free vaccine program for 12-year-old girls in Korea as of 2016, will likely prevent future cases of cervical cancer. However, current commercial HPV vaccines showed limited effects. Gardasil (Merck, NJ, USA) showed limited effects against specific viruses including HPV 6, 11, 16, and 18. Gardasil 9 (Merck) showed limited effects against HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58, while Cervarix (GSK, London, UK) showed limited effects against HPV 16 and 18. HPV types 16 and 18 account for between 55% and 70% of cancers and mainly cause cervical cancers. At least 30% of cancers contain other HR-HPV types and would not be prevented by current vaccines.

Thus, even if HPV vaccines become publicly available, both non-vaccinated women and vaccinated women will need to undergo regular cervical screening using the best, most sensitive and specific available screening tools available in the current era. Moreover, current commercial HPV vaccines have no effect against the prevalent HR-HPV 53/66 and LR-HPV 70 strains in cohort studies, thus demonstrating limitations to the application of commercial HPV vaccines in Korea. Thus, a more effective HPV vaccine must be developed in Korea, based on epidemiological studies of larger Korean population and regional samples.

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**Conflicts of Interest**

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Cervical Cancer Screening in Korea


Association between Expression of 8-OHdG and Cigarette Smoking in Non-small Cell Lung Cancer

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Background: Exposure to cigarette smoking (CS) is a major risk factor for the development of lung cancer. CS is known to cause oxidative DNA damage and mutation of tumor-related genes, and these factors are involved in carcinogenesis. 8-Hydroxydeoxyguanosine (8-OHdG) is considered to be a reliable biomarker for oxidative DNA damage. Increased levels of 8-OHdG are associated with a number of pathological conditions, including cancer. There are no reports on the expression of 8-OHdG by immunohistochemistry in non-small cell lung cancer (NSCLC).

Methods: We investigated the expression of 8-OHdG and p53 in 203 NSCLC tissues using immunohistochemistry and correlated it with clinicopathological features including smoking.

Results: The expression of 8-OHdG was observed in 83.3% of NSCLC. It was significantly correlated with a low T category, negative lymph node status, never-smoker, and longer overall survival (p < .05) by univariate analysis. But multivariate analysis revealed that 8-OHdG was not an independent prognostic factor for overall survival in NSCLC patients. The aberrant expression of p53 significantly correlated with smoking, male, squamous cell carcinoma, and Ki-67 positivity (p < .05).

Conclusions: The expression of 8-OHdG was associated with good prognostic factors. It was positively correlated with never-smokers in NSCLC, suggesting that oxidative damage of DNA cannot be explained by smoking alone and may depend on complex control mechanisms.

Key Words: Carcinoma, non-small cell lung; 8-Oxo-7-hydrodeoxyguanosine; p53; Smoking; Immunohistochemistry

Lung cancer has been the most common cancer in the world for several decades and remains the leading cause of cancer deaths worldwide.1 Exposure to cigarette smoking (CS) is a major risk factor for the development of lung cancer.1 However, the exact mechanism by which CS is involved in lung cancer development is still unclear. Smoking is known as a major inducer of oxidative stress, which in turn is known to cause DNA damage and mutation of tumor-related genes, and these factors are involved in carcinogenesis.

Oxidative damage of DNA by reactive oxygen and nitrogen species leads to the production of 8-hydroxy-2-deoxy guanosine (8-OHdG), which is a specific biomarker of oxidative stress. Increased levels of 8-OHdG are associated with the aging process as well as a number of pathological conditions, including cancer, diabetes, and hypertension.2 Previous studies reported that increased 8-OHdG expression in cancer was associated with a poor prognosis.3 However, the clinical significance of 8-OHdG expression as a biomarker is still controversial. Negative 8-OHdG expression was associated with an aggressive cancer phenotype.4 Also, 8-OHdG has been measured in urine or serum rather than in cancer tissue, and there is no report of the expression of 8-OHdG by immunohistochemistry of non-small cell lung cancer (NSCLC). Therefore, we thought that it would be meaningful to measure 8-OHdG in cancer tissues by immunohistochemistry and to examine the relationship between their expression and clinicopathologic factors.

p53 is an important transcription factor regulating intracellular pathways related to cell survival, DNA-repair, and apoptosis. TP53 mutations are one of the most common mutations found in lung cancers and occur more frequently in smoking-
related lung cancer. It is known that the common types of cancer-associated TP53 mutations not only lose tumor suppression function but acquire a function related to tumor progression, such as cell-cycle progression or cell migration. This leads to malignant transformation and cancer progression. Oxidative stress is known to play an important role in p53 mutations caused by smoking. It is known that impaired capability of mutant p53 to promote DNA repair leads to cancer development. Previous studies showed that positive immunoreactivity for 8-OHdG was more frequent in cases positive for p53 in malignant melanoma.

To better understand the role of oxidative damage in carcinogenesis in lung cancer, we examined the expression of 8-OHdG in non-small cell lung cancer using immunohistochemistry and correlated with clinicopathologic features including smoking and p53 expression.

MATERIALS AND METHODS

Patients and follow-up

This study evaluated NSCLC patients who underwent surgical resection between January 2007 and December 2012. Among them, 203 NSCLC had complete available medical records, original hematoxylin and eosin slides, and paraffin-embedded tissue blocks. Clinical information including smoking history and postoperative follow-up was obtained by reviewing the medical records. The pathologic diagnosis and staging were reviewed according to the 8th American Joint Committee on Cancer staging system and World Health Organization classification.7 The classification of smoking status follows the Centers for Disease Control and Prevention guidelines, and the status categories were “Never,” “Former” (adults who have smoked at least 100 cigarettes in their lifetime, but say they currently do not smoke), or “Current” (adults who have smoked 100 cigarettes in their lifetime and currently smoke cigarettes). This study was approved by the institutional review board of Chonbuk National University Hospital (CUH 2017-09-029-001). All samples derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols.

Immunohistochemical staining and scoring

Formalin-fixed paraffin-embedded tissue of NSCLC patients were used to establish tissue microarrays. Original hematoxylin and eosin slides were reviewed, and one 3.0-mm-sized core from the representative area was taken. The tissue sections were de-paraffinized and boiled in an antigen retrieval solution (citrate, pH 6.0) for 20 minutes in a cooker. The primary antibodies for 8-OHdG (1:200, N45.1, JaiCA, Shizuoka, Japan), p53 (1:800, DO-7, Leica, London, UK), and Ki-67 (1:100, MIB-1, Dako, Glostrup, Denmark) were used for immunohistochemical staining. The slides that had been stained for 8-OHdG, p53, and Ki-67 were evaluated by two pathologists (M.J.C. and A.R.A.) who had no clinicopathologic information on the patients. Immunohistochemical staining was evaluated to estimate the nuclear positivity of tumor cells for each marker, and the stained area was semiquantitatively recorded for the area of positive cells in 5% increments.

Immunohistochemical staining for 8-OHdG and Ki-67 was considered positive when ≥ 5% and ≥ 10% of the tumor cells showed moderate to strong nuclear staining, respectively. The cut-off points for the immunohistochemical staining score were determined by receiver operating characteristic (ROC) curve analysis. It has recently been reported that immunohistochemical staining for p53 can be used as a surrogate for TP53 mutation analysis.8,9 Complete negative and high expression showed strong correlation with TP53 mutation.8,9 We categorized immunohistochemical results for p53 into three groups as negative, low (1%–50% positively stained nuclei), and high (>50% positively stained nuclei) as previously reported.10 Then, p53 immunohistochemical staining was defined as “aberrant expression” in the p53 negative and high expression group and “wild type expression” in the p53 low expression group.

Statistical analysis

ROC curve analysis was used to determine the cut-off points for immunohistochemical staining. The cutoff points for 8-OHdG– and Ki-67–positivity were determined at the point with the highest area under the curve (AUC) to estimate survival of NSCLC patients. Because the ROC curve is a plot of the true positive rate (sensitivity) versus the false positive rate (1–specificity) for determination of patient death, the cutoff level for the ideal test is presented as sensitivity 1 and specificity 1 (AUC, 1.000). Therefore, we chose the cut-off point at the highest AUC value.

The date of last follow-up was recorded as the last contact or the death of patients through August 2017. The prognosis of NSCLC patients was evaluated by analyzing overall survival (OS). The death of the patient from lung cancer was considered an event for OS analysis. SPSS software ver. 22.0 (IBM Corp., Armonk, NY, USA) was used to perform statistical analysis for the univariate and multivariate Cox proportional hazards regression analyses, Kaplan-Meier survival analysis, and Pearson’s chi-square test. The
Correlations between 8-OHdG expression and clinicopathologic parameters of NSCLC patients

In this study, the NSCLC used consisted of 94 squamous cell carcinomas and 109 adenocarcinomas. The mean age of the study subjects was 65 years. In all, 72.4% of the subjects were male, and 27.3% were female. Expression of 8-OHdG was found exclusively in nuclei of tumor cells and was positive in 83.3% (169/203) of NSCLC samples (Table 1, Fig. 1). The positive expression of 8-OHdG was significantly associated with female, adenocarcinoma, smaller tumor size, lower T group, negative lymph node status, and never-smoker (p < .05) (Table 1). The expression of 8-OHdG was high in never-smoker (89.5%) compared to smoker (73.4%) (p = .003).

For p53 immunohistochemistry, 18% (37/203), 30% (60/203), and 52% (106/203) of cases were categorized as p53 negative, p53 low, and p53 high groups, respectively. Analysis was performed on the correlations between p53 staining pattern and clinicopathological features of NSCLC patients, and p53 aberrant expression was significantly correlated with male, smoker, squamous cell carcinoma, and Ki-67 positivity (p < .05) (Table 2). The p53 wild-type expression was high in never-smoker (39.5%) compared to smoker (13.9%), and p53 aberrant expression was 86.1% (68/79) in smoker and 60.5% (75/124) in never-smoker (p < .001). The p53 aberrant expression rate was 87.2% in squamous cell carcinoma and 56% in lung adenocarcinoma. In the 8-OHdG negative group, p53 aberrant expression pattern was more frequent (82.4%, 28/34) than in the 8-OHdG positive group (68%, 115/169) but not to an extent that was statistically significant (p = .095). Comparing the p53 wild-type and the p53 aberrant patients, there was no significant difference in tumor stage or lymph node stage.

**Table 1.** Clinicopathologic characteristics and the expression of 8-OHdG in non-small cell lung carcinomas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>8-OHdG (+)</th>
<th>8-OHdG (-)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 65</td>
<td>84</td>
<td>71 (84.5)</td>
<td>13 (15.5)</td>
<td>.683</td>
</tr>
<tr>
<td>≥ 65</td>
<td>119</td>
<td>98 (82.4)</td>
<td>21 (17.6)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>144</td>
<td>115 (79.9)</td>
<td>29 (20.1)</td>
<td>.030</td>
</tr>
<tr>
<td>Female</td>
<td>59</td>
<td>54 (91.5)</td>
<td>5 (8.5)</td>
<td></td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SqCC</td>
<td>94</td>
<td>73 (77.7)</td>
<td>21 (22.3)</td>
<td>.037</td>
</tr>
<tr>
<td>ADC</td>
<td>109</td>
<td>96 (89.1)</td>
<td>13 (10.9)</td>
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</tr>
<tr>
<td>T category</td>
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</tr>
<tr>
<td>1 + 2</td>
<td>119</td>
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<td>10 (8.4)</td>
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<td>3 + 4</td>
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<td>60 (71.4)</td>
<td>24 (28.6)</td>
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<tr>
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<td>117 (86.7)</td>
<td>18 (13.3)</td>
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<tr>
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<td>68</td>
<td>52 (76.5)</td>
<td>16 (23.5)</td>
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</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
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<td>.003</td>
</tr>
<tr>
<td>Former + current</td>
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<td>58 (73.4)</td>
<td>21 (26.6)</td>
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</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Wild-type</td>
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<tr>
<td>Aberrant</td>
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<td>115 (80.4)</td>
<td>28 (19.6)</td>
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</tr>
<tr>
<td>Ki-67</td>
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<td>47</td>
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<tr>
<td>Positive</td>
<td>156</td>
<td>126 (80.8)</td>
<td>30 (19.2)</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as number (%).
8-OHdG, 8-hydroxy-2-deoxy guanosine; SqCC, squamous cell carcinoma; ADC, adenocarcinoma.

NSCLC patients with negative 8-OHdG expression had shorter OS by univariate analysis

When univariate Cox proportional regression analysis was performed for the survival of NSCLC patients, older age (≥ 65), male, smoker, higher tumor stage, presence of lymph node metastasis, 8-OHdG negativity and p53 aberrant expression, and Ki-67 positivity showed significant association with shorter OS of NSCLC patients by univariate analysis (Table 3). The patients with 8-OHdG negativity showed 1.847-fold (95% confidence interval [95% CI], 1.054 to 3.236; p = .032) greater risk of death. Smoking (former and current) predicted a 3.926-fold (95% CI, 2.302 to 6.697; p < .001) greater risk of death than the never-smoker group. The p53 aberrant expression group predicted a 2.031-fold (95% CI, 1.057 to 3.900; p = .033) greater risk of death than the p53 wild-type group. Kaplan-Meier survival analysis was used to analyze the prognostic impact of the expression of 8-OHdG, p53, and Ki-67 and smoking and tumor stage on OS, and the results are presented in Fig. 2.

The multivariate analysis included the factors that showed p-value lower than .05 in univariate analysis (sex, age, smoking, T category, lymph node stage, p53 expression, Ki-67 expression, and 8-OHdG expression). Age older than 65, smoker, and higher T category in the TNM stage system were independent poor prognostic factors for OS of NSCLC patients (Table 4). However, multivariate analysis did not indicate that 8-OHdG expression was an independent prognostic factor associated with OS for NSCLC.
DISCUSSION

In this study, we investigated the immunohistochemical expression of 8-OHdG and p53 in human NSCLC tissues. Our results show that (1) expression of 8-OHdG was observed in 83.3% of NSCLC tissue; (2) expression of 8-OHdG was significantly associated with low T category, negative lymph node status, and never-smoker; (3) expression of 8-OHdG was significantly associated with longer OS by univariate and Kaplan-Meier survival analysis; (4) p53 aberrant expression closely correlated with smoker, male, squamous cell carcinoma, and Ki-67 positivity.

CS induces oxidative stress, which can cause severe DNA damage and is known as an important carcinogenic mechanism linked to NSCLC. It is challenging to perform a direct measurement of reactive oxygen species (ROS) because of their short lifetime and rapid degradation and removal by cellular defense mechanisms. Therefore, a useful method to assess ROS is to use antibodies against the specific “footprints” of oxidative damage. The oxidative damage of DNA by ROS produces 8-OHdG, and this serves as an established footprint of oxidative stress. Increased levels of 8-OHdG are associated with the aging process as well as with a number of pathological conditions including cancer, diabetes, and hypertension.

The overexpression of 8-OHdG has been reported in a variety of cancers, including breast cancer, lung cancer, bladder cancer, colorectal cancer, renal cell carcinoma, prostate cancer, and gastric adenocarcinoma. Reports have predominantly indicated that overexpression of 8-OHdG is associated with unfavorable prognostic factors and/or poor patient prognosis. However, some studies reported different results. Negative 8-OHdG expression was associated with an aggressive breast cancer phenotype, and some reports have found no difference in 8-OHdG level between cancer patients and healthy subjects. Therefore, there is controversy surrounding the clinical significance of 8-OHdG expression. Our results showed that tumors with positive expression of 8-OHdG were significantly associated with favorable clinicopathologic factors and longer OS in NSCLC patients. We considered the following possibilities as the cause of the different results. In many of the previous studies, 8-OHdG was measured in urine or at the serum level, not in cancer tissue. It is known that the method of measuring the absolute amount of 8-OHdG in urine or serum can be influenced by artificial production of 8-OHdG in the sample preparation stage. Also, a previous study showed that serum and urinary levels of 8-OHdG did not associate with those found in the tissues. We detected 8-OHdG expression using immunohistochemistry because immunohistochemical procedures allow detection of 8-OHdG at the single-cell level, which has the advantage of directly confirming its expression in cancer cells in tissues composed of different cell populations. To the best of our knowledge, this is the first study to use...
immunohistochemistry to investigate the correlation between 8-OHdG expression and prognosis in NSCLC patients.

Several possible mechanisms may be behind the inverse association of 8-OHdG level and tumor aggressiveness. Sova et al. have insisted that the increase in ROS induces the overproduction of antioxidant enzymes, thereby avoiding the death of tumor cells and inducing tumor growth. Overproduction of antioxidant enzymes would prevent ROS interaction with DNA, leading to decreased formation of 8-OHdG at the tissue level, as suggested by their results. Another reasonable possibility we suggest is that cancer tissue is generally hypoxic, which is predicted to be the reason for low ROS in cancer cells, because oxygen is needed for production of ROS. Thus, ROS production is low in rapidly growing cancers, leading to a decrease in the production of 8-OHdG, which may explain the poor prognosis of negative 8-OHdG in NSCLC patients in this study. Although there was no statistical significance, the association between negative 8-OHdG and negative Ki-67 (p = .084) in this study may support this hypothesis. Previous studies showed that ROS-mediated DNA damage plays a role in the initiation of carcinogenesis as well as in malignant transformation. The expression of 8-OHdG decreased significantly in invasive breast carcinomas compared to non-invasive lesions, namely usual ductal hyperplasia, atypical ductal hyperplasia, and ductal carcinoma in situ. To support the hypothesis that ROS-mediated DNA damage may be involved in the early stages of cancer development rather than cancer progression, examination of 8-OHdG expression in pre-cancerous lesions or pre-invasive lung cancer lesions is necessary. In addition, a DNA repair enzyme assessment is needed to clarify the fundamental background behind the current results.

In this study, expression of 8-OHdG was significantly associated with never-smoker (p = .003). We cannot explain the exact mechanism for the relationship between never-smokers and 8-OHdG expression in this study. However, we inferred some possibilities based on the results of other researchers. The formation of carboxyhemoglobin in smokers can lower the ability of hemoglobin to deliver O₂ (toxic hypoxia) and result in hypoxia. In turn, this can reduce ROS formation and lower the expression of 8-OHdG. The association between smoking and low 8-OHdG may also be explained by smoking-induced oxidative stress, i.e., overproduction of antioxidant enzymes. The state of the smoking-ROS-8-OHdG axis in the mechanism of NSCLC development by smoking is thought to be regulated by more complex regulatory mechanisms, including the functional status of ROS and antioxidant enzymes and the hypoxic environment of cancer.

DNA adduct formation and oncogenic mutation in lung epithelial cells are caused by chronic exposure to carcinogens in CS, and the accumulation of mutations is known to be involved in lung cancer development. TP53 mutation is an important muta-

**Table 2.** Clinicopathologic characteristics and the expression of p53 in non-small cell lung carcinomas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>p53</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Aberrant</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 65</td>
<td>84</td>
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<tr>
<td>≥ 65</td>
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<td>36 (30.3)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>144</td>
<td>32 (22.2)</td>
</tr>
<tr>
<td>Female</td>
<td>59</td>
<td>28 (47.5)</td>
</tr>
<tr>
<td>Histologic type</td>
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</tr>
<tr>
<td>SqCC</td>
<td>94</td>
<td>12 (12.8)</td>
</tr>
<tr>
<td>ADC</td>
<td>109</td>
<td>48 (44.0)</td>
</tr>
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<td>T category 8th</td>
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<tr>
<td>1 + 2</td>
<td>119</td>
<td>40 (33.6)</td>
</tr>
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<td>3 + 4</td>
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<td>20 (23.8)</td>
</tr>
<tr>
<td>N category</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>108</td>
<td>36 (33.3)</td>
</tr>
<tr>
<td>N1</td>
<td>95</td>
<td>24 (25.3)</td>
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<td></td>
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<td>Negative</td>
<td>135</td>
<td>41 (30.4)</td>
</tr>
<tr>
<td>Positive</td>
<td>68</td>
<td>19 (27.9)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>124</td>
<td>49 (39.5)</td>
</tr>
<tr>
<td>Former + current</td>
<td>79</td>
<td>11 (13.9)</td>
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<td>8-OHdG</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>34</td>
<td>6 (17.6)</td>
</tr>
<tr>
<td>Positive</td>
<td>169</td>
<td>54 (32.0)</td>
</tr>
<tr>
<td>Ki-67</td>
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<td></td>
</tr>
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<td>Negative</td>
<td>47</td>
<td>22 (46.8)</td>
</tr>
<tr>
<td>Positive</td>
<td>156</td>
<td>38 (24.4)</td>
</tr>
</tbody>
</table>

Values are presented as number (%). SqCC, squamous cell carcinoma; ADC, adenocarcinoma; 8-OHdG, 8-hydroxy-2-deoxy guanosine.

**Table 3.** Univariate Cox regression analysis for the survival of NSCLC patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>OS (n=203)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI) p-value</td>
</tr>
<tr>
<td>Age, ≥ 65 yr (vs &lt; 65 yr)</td>
<td>2.153 (1.231–3.764)</td>
</tr>
<tr>
<td>Sex, female (vs male)</td>
<td>0.324 (0.154–0.681)</td>
</tr>
<tr>
<td>T category, T3+4 (vs 1 + 2)</td>
<td>4.027 (2.318–6.993)</td>
</tr>
<tr>
<td>N category, N1 (vs N0)</td>
<td>3.620 (2.042–6.418)</td>
</tr>
<tr>
<td>Pleural invasion, positive (vs negative)</td>
<td>1.403 (0.844–2.331)</td>
</tr>
<tr>
<td>Smoking, former + current (vs never)</td>
<td>3.926 (2.302–6.697)</td>
</tr>
<tr>
<td>8-OHdG negative (vs positive)</td>
<td>1.847 (1.054–3.236)</td>
</tr>
<tr>
<td>p53 aberrant (vs wild-type)</td>
<td>2.031 (1.057–3.900)</td>
</tr>
<tr>
<td>Ki-67 positive (vs negative)</td>
<td>4.982 (1.808–13.731)</td>
</tr>
</tbody>
</table>

NSCLC, non-small cell lung cancer; 8-OHdG, 8-hydroxy-2-deoxy guanosine.
An AR et al. • 8-OHdG and p53 in NSCLC and its relationship with clinicopathologic factors and patient survival. Univariate analysis revealed that expression of 8-OHdG was significantly associated with longer OS. Smoking is an important inducer of oxidative stress, but it was not associated with increased expression of 8-OHdG in this study, suggesting that more complex regulatory mechanisms regulate 8-OHdG expression in cancer, including the functional status of the antioxidant enzyme and the hypoxic environment of cancer. The aberrant expression patterns of p53

Table 4. Multivariate Cox regression analysis for the survival of NSCLC patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T category, T 3,4 (vs 1,2)</td>
<td>2.346 (1.267–4.343)</td>
<td>.007</td>
</tr>
<tr>
<td>Smoking, former + current (vs never)</td>
<td>2.371 (1.347–4.172)</td>
<td>.003</td>
</tr>
<tr>
<td>Age, ≥ 65 yr (vs &lt; 65 yr)</td>
<td>2.154 (1.220–3.604)</td>
<td>.03</td>
</tr>
<tr>
<td>8-OHdG negative (vs positive)</td>
<td>1.486 (0.520–4.248)</td>
<td>.460</td>
</tr>
<tr>
<td>p53 aberrant (vs wild-type)</td>
<td>1.211 (0.671–2.184)</td>
<td>.525</td>
</tr>
</tbody>
</table>

Variables considered in the model were age, lymph node status, smoking history, T category (American Joint Committee on Cancer 8th edition), 8-OHdG expression and p53, Ki-67 expression.

NSCLC, non-small cell lung cancer; 8-OHdG, 8-hydroxy-2-deoxyguanosine.

The p53 aberrant expression rate was 87.2% in squamous cell carcinoma and 56% in lung adenocarcinoma (data not shown). Our results are consistent with another report that found p53 mutations in 46% of lung adenocarcinomas and 81% of squamous cell carcinomas. The p53 aberrant expression is closely correlated with smoker (p = .000), male (p = .000), squamous cell carcinoma (p = .000), and Ki-67 positivity (p = .003). Further studies are needed to determine the concordance of p53 immunohistochemistry with TP53 mutations in NSCLC to confirm our assertion.

In conclusion, this study has demonstrated the expression of 8-OHdG and p53 in NSCLC and its relationship with clinicopathologic factors and patient survival. The p53 aberrant expression rate was 87.2% in squamous cell carcinoma and 56% in lung adenocarcinoma (data not shown). Our results are consistent with another report that found p53 mutations in 46% of lung adenocarcinomas and 81% of squamous cell carcinomas. The p53 aberrant expression is closely correlated with smoker (p = .000), male (p = .000), squamous cell carcinoma (p = .000), and Ki-67 positivity (p = .003). Further studies are needed to determine the concordance of p53 immunohistochemistry with TP53 mutations in NSCLC to confirm our assertion.

In conclusion, this study has demonstrated the expression of 8-OHdG and p53 in NSCLC and its relationship with clinicopathologic factors and patient survival. Univariate analysis revealed that expression of 8-OHdG was significantly associated with longer OS. Smoking is an important inducer of oxidative stress, but it was not associated with increased expression of 8-OHdG in this study, suggesting that more complex regulatory mechanisms regulate 8-OHdG expression in cancer, including the functional status of the antioxidant enzyme and the hypoxic environment of cancer. The aberrant expression patterns of p53

Fig. 2. Kaplan-Meier survival analysis of non-small cell lung carcinoma patients. Overall survival in 203 patients according to the expression of 8-hydroxydeoxyguanosine (8-OHdG) (A), p53 (B), Ki-67 (C), smoking history (D), and T category (American Joint Committee on Cancer (AJCC) 8th edition) (E).

Table 4. Multivariate Cox regression analysis for the survival of NSCLC patients
immunohistochemical staining showed significant associations with smoking status.

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

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REFERENCES


CpG Island Methylation in Sessile Serrated Adenoma/Polyp of the Colorectum: Implications for Differential Diagnosis of Molecularly High-Risk Lesions among Non-dysplastic Sessile Serrated Adenomas/Polyps

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Background: Although colorectal sessile serrated adenomas/polyps (SSA/Ps) with morphologic dysplasia are regarded as definite high-risk premalignant lesions, no reliable grading or risk-stratifying system exists for non-dysplastic SSA/Ps. The accumulation of CpG island methylation is a molecular hallmark of progression of SSA/Ps. Thus, we decided to classify non-dysplastic SSA/Ps into risk subgroups based on the extent of CpG island methylation. Methods: The CpG island methylator phenotype (CIMP) status of 132 non-dysplastic SSA/Ps was determined using eight CIMP-specific promoter markers. SSA/Ps with CIMP-high and/or MLH1 promoter methylation were regarded as a high-risk subgroup. Results: Based on the CIMP analysis results, methylation frequency of each CIMP marker suggested a sequential pattern of CpG island methylation during progression of SSA/P, indicating MLH1 as a late-methylated marker. Among the 132 non-dysplastic SSA/Ps, 34 (26%) were determined to be high-risk lesions (33 CIMP-high and 8 MLH1-methylated cases; seven cases overlapped). All 34 high-risk SSA/Ps were located exclusively in the proximal colon (100%, p = .001) and were significantly associated with older age (≥ 50 years, 100%; p = .003) and a larger histologically measured lesion size (> 5 mm, 100%; p = .004). In addition, the high-risk SSA/Ps were characterized by a relatively higher number of typical base-dilated serrated crypts. Conclusions: Both CIMP-high and MLH1 methylation are late-step molecular events during progression of SSA/Ps and rarely occur in SSA/Ps of young patients. Comprehensive consideration of age (≥ 50), location (proximal colon), and histologic size (> 5 mm) may be important for the prediction of high-risk lesions among non-dysplastic SSA/Ps.

Key Words: Colorectal neoplasms; DNA methylation; Serrated lesion; Serrated pathway; Serrated polyp

It is widely known that there are two morphologic multistep colorectal carcinogenesis pathways: the conventional pathway and the serrated pathway.1,2 In the conventional pathway, colorectal carcinomas (CRCs) develop through premalignant lesions, including tubular, tubulovillous, and villous adenomas, accounting for approximately 60%–80% of the CRCs, whereas in the serrated pathway, about 15%–35% of the CRCs progress from serrated precursor lesions.1,3,4 According to the latest (4th) edition of the World Health Organization (WHO) classification of tumors of the digestive system, serrated colorectal lesions are classified into three categories: hyperplastic polyps (HPs), sessile serrated adenomas/polyps (SSA/Ps), and traditional serrated adenomas (TSAs).5 Among the three subtypes of serrated lesions, SSA/Ps and TSAs are recognized as premalignant lesions, whereas HPs are not regarded as direct precursors of carcinoma but rather as potential precursors of SSA/Ps or TSAs.6,7

SSA/Ps are considered major precursors of the CpG island methylator phenotype-high (CIMP-high) CRCs.5,6,8 Histologically, SSA/Ps are typically characterized by distorted crypt architecture, including horizontal extension (inverted T- or L-shape) and branching of the lumen at the crypt bases.5,6 Through genetic and epigenetic alterations, they can acquire cytologic dysplasia and ultimately progress toward carcinoma. The typical genetic and epigenetic characteristics of SSA/Ps are BRAF V600E mutation and CpG island hypermethylation, respectively.5,6

It has been suggested that risk factors to predict the progression of SSA/Ps are morphologic dysplasia, size of the polyp, and the number of serrated polyps.6,10 Based on these potential risk...
factors, endoscopic surveillance guidelines have generally recommended shorter surveillance interval for SSA/Ps with morphologic dysplasia, large polyp size (≥ 10 mm), or multiple SSA/Ps or TSAs (3 or more). However, although SSA/Ps without dysplasia are more prevalent than SSA/Ps with dysplasia, there has been a lack of reliable grading or risk-stratifying system for non-dysplastic SSA/Ps. Therefore, identifying clinicopathologic factors correlated with underlying molecular alterations that indicate high-risk lesions would be important for establishing a precise risk-assessment system for non-dysplastic SSA/Ps.

The accumulation of CpG island methylation is known as a molecular hallmark of the progression of SSA/Ps. Thus, CIMP-high and MLH1 methylation status can be strong molecular indicators of the high potential of malignant change in SSA/Ps. In this study, DNA methylation status of non-dysplastic SSA/Ps was analyzed using DNA extracted from the lesions, and non-dysplastic SSA/Ps were classified into two risk subgroups based on the CIMP status: high-risk (CIMP-high and/or MLH1 promoter hypermethylation) and low-risk (CIMP-low/negative) subgroups. We then aimed to investigate the differential clinicopathologic characteristics between high-risk and low-risk subgroups. Through this analysis, we expect to find clinicopathologic factors that can aid in the prediction of molecularly high-risk lesions among non-dysplastic SSA/Ps.

**MATERIALS AND METHODS**

**Tissue samples**

We initially reviewed a total of 275 colorectal polyps diagnosed with the term “sessile serrated adenoma” or “sessile serrated polyp,” all of which had been removed by endoscopic mucosal resection or polypectomy between 2010 and 2012 at Seoul National University Hospital, Seoul, Korea. The recommendations proposed by an American expert panel in 2012 were used to diagnose SSA/Ps in our study. Of these 275 cases, 234 were determined to be morphologically definite SSA/Ps through independent microscopic review by three gastrointestinal pathologists (J.A.L., H.E.P., and J.H.K.) Finally, 133 cases were selected as potential pure forms of sporadic non-dysplastic SSA/Ps, based on the strict inclusion and exclusion criteria of this study, listed below:

- Inclusion criteria: presence of one or more typical distorted crypt for SSA/P (dilated and/or horizontally branched crypt base) in the lesion
- Exclusion criteria: satisfying one of the WHO diagnostic criteria for serrated polyposis, presence of TSA or conventional adenoma component in the lesion (mixed polyp), and presence of morphologic dysplasia in the lesion

This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 1804-109-939). Under the condition of retrospective archival tissue collection and patient data anonyzation, our study was exempted from the acquisition of informed consent from patients.

**Clinical data collection and histomorphometric analysis**

Clinical data, including the age and sex of patients, size and location of non-dysplastic SSA/Ps, and number and diagnosis of synchronous polyps, were collected by reviewing the electronic medical records. Sidedness of the location of each SSA/P was categorized into proximal or distal using splenic flexure as the cutoff site. Multiplicity of serrated lesions was determined by counting the total number of polyps diagnosed as HP, SSA/P, TSA or serrated polyp (unclassified), which were biopsied or resected during the same endoscopic evaluation. In addition to the endoscopically measured size of non-dysplastic SSA/Ps, we measured the maximum size of each SSA/P histologically using the Aperio ImageScope program (ver. 12.0.0., Leica Biosystems, Nussloch, Germany) (Fig. 1A). Other quantitative histomorphometric analyses, including the total number and the largest diameter of typical base-dilated serrated crypts in each SSA/P, were also performed using the Aperio ImageScope program (Fig. 1B, C).

**DNA methylation analysis**

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue blocks of 133 non-dysplastic SSA/Ps. After the extraction of genomic DNA, the DNA quantity of one of the 133 SSA/Ps was found to be insufficient for DNA experiments, and therefore, a total of 132 non-dysplastic SSA/Ps were finally subjected to DNA methylation analysis. Bisulfite modification and CIMP analysis of the 132 DNA samples were conducted as previously described. For CIMP determination, methylation-specific quantitative polymerase chain reaction (PCR) analysis (MethyLight assay) was performed using eight CIMP-specific promoter markers (MLH1, CAGNA1G, CDKN2A (p16), CRABP1, IGF2, NEUROG1, RUNX3, and SOCS1). A CIMP marker was defined as hypermethylated when the percentage of methylated reference (PMR) value was 4 or more. Samples were classified as CIMP-high if they showed CpG island hypermethylation in five or more CIMP markers, and as CIMP-low if one to four CIMP markers were hypermethylated. CIMP-negative indicated that there was no hypermethylated marker in the sample. The classification criteria, marker panel, and PMR cut-
off value for CIMP determination used in this study were based on the methodology developed by Dr. Shuji Ogino that had been validated by previous studies.\(^{15-18}\) The methylation levels of long interspersed nucleotide element-1 (LINE-1) were also measured in 132 non-dysplastic SSA/Ps using pyrosequencing assay. The primers and PCR conditions were used as described previously.\(^{19}\)

**Immunohistochemistry**

Immunohistochemistry (IHC) for MLH1 was performed on whole FFPE tissue sections of 132 non-dysplastic SSA/Ps using the Ventana Benchmark XT automated immunostainer (Roche, Basel, Switzerland) with a primary antibody against MLH1 (clone M1, Ventana RTU, Roche).

**Statistical analysis**

Statistical analyses in this study were performed using the IBM SPSS Statistics ver. 23 (IBM Corp., Armonk, NY, USA). Comparisons of categorical variables were conducted using the chi-square test or Fisher exact test. Comparisons of continuous variables were conducted using the Student’s t test or Mann-Whitney U test. All p-values were two-sided, and the statistical significance was determined at \(p < .05\).

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**RESULTS**

**Developmental pattern of CpG island methylation in SSA/Ps**

Among 132 non-dysplastic SSA/Ps, the number of CIMP-high, CIMP-low, and CIMP-negative cases were 33 (25%), 73 (55%), and 26 (20%), respectively. First, to infer a developmental pattern of CpG island methylation during progression of SSA/Ps, we performed in-depth analyses using methylation results of each CIMP marker in the 132 SSA/Ps (Fig. 2, Supplementary Fig. S1). Based on the number of concurrently methylated markers, eight CIMP markers were classified into three groups (Fig. 2A, B). Group A markers, including \(\text{CRABP1}^{\text{p16}}\), \(\text{NEUROG1}^{\text{p16}}\), and \(\text{CDKN2A}^{\text{p16}}\), represented early-methylated markers in the serrated neoplasia pathway, because they could be methylated without any other concurrently methylated marker in SSA/Ps. In contrast, group C markers, including \(\text{SOCS1}\) and \(\text{MLH1}\), represented late-methylated markers, because they could not be methylated without at least two or three concurrently methylated markers (Fig. 2A). Group B markers, including \(\text{CACNA1G}\), \(\text{IGF2}\), and \(\text{RUNX3}\), could be methylated when only one other marker was methylated, but not when there was no other concurrently methylated marker (Fig. 2A). Thus, \(\text{CACNA1G}\), \(\text{IGF2}\), and \(\text{RUNX3}\) could be regarded as intermediately meth-
ylated markers in the progression pathway of SSA/Ps (Fig. 2B). In addition, the characteristic sequential pattern of methylation of CIMP markers in SSA/Ps was closely associated with age of the patients. We further analyzed the percentages of methylation-positive cases for each CIMP marker in the 132 SSA/Ps, according to age subgroups (Supplementary Fig. S1). We found that

Fig. 2. An inference of developmental pattern of CpG island methylation in colorectal serrated neoplasia pathway. (A) Frequencies of methylation-positive cases for each CpG island methylator phenotype (CIMP) marker, based on the number of concurrently methylated markers in 132 non-dysplastic sessile serrated adenomas/polyps (SSA/Ps). Group A markers can be methylated independently and alone, whereas methylation of group C markers accompanies at least two or three other methylated markers. (B) A sequence model of methylation of CIMP markers during progression of SSA/Ps.
CRABP1 and NEUROG1, the early-methylated markers, were methylated even in the younger age group (< 40) (Supplementary Fig. S1A, S1B), whereas SOCS1 and MLH1, the late-methylated markers, were not methylated before the age of 50 (Supplementary Fig. S1G, S1H).

**MLH1 expression status in MLH1-methylated SSA/Ps**

Eight of 132 non-dysplastic SSA/Ps (6%) were determined to be MLH1-methylated. Using IHC, we evaluated the MLH1 protein expression status in eight MLH1-methylated non-dysplastic SSA/Ps. Surprisingly, among the eight MLH1-methylated non-dysplastic SSA/Ps, there was no case with a complete loss of MLH1 expression (Supplementary Fig. S2). To compare the differential features between non-dysplastic and dysplastic SSA/Ps harboring MLH1 methylation, we analyzed two additional cases confirmed as MLH1-methylated SSA/Ps with morphologic dysplasia (Supplementary Table S1). In contrast to the MLH1-methylated non-dysplastic SSA/Ps, both these MLH1-methylated dysplastic SSA/Ps demonstrated a complete loss of MLH1 expression (Supplementary Table S1, Supplementary Fig. S2D). However, only one of the eight MLH1-methylated non-dysplastic SSA/Ps showed a partial loss of MLH1 expression (Supplementary Table S1, Supplementary Fig. S2B, S2C). The MLH1 promoter PMR value (26.3) for this MLH1-partial-deficient case was greater than that of the other MLH1-methylated non-dysplastic SSA/Ps (5 to 12.9), but less than that of MLH1-methylated dysplastic SSA/Ps (36.04 to 57.07) (Supplementary Table S1). Therefore, it could be interpreted that MLH1 methylation found in non-dysplastic SSA/Ps represents subrepressive, low-level methylation. It is also notable that MLH1-methylated dysplastic SSA/Ps, as well as MLH1-methylated non-dysplastic SSA/Ps, were found exclusively in individuals aged 50 years or more (Supplementary Table S1).

**Clinicopathologic factors associated with high-risk subgroup of non-dysplastic SSA/Ps**

In this study, we defined the high-risk subgroup of SSA/Ps as lesions harboring CIMP-high and/or MLH1 promoter methylation. As mentioned above, 33 of 132 non-dysplastic SSA/Ps (25%) were CIMP-high lesions, and eight of 132 non-dysplastic SSA/Ps (6%) demonstrated MLH1 methylation. Seven of the eight MLH1-methylated SSA/Ps overlapped with CIMP-high cases. Thus, among the 132 non-dysplastic SSA/Ps, 34 cases (26%) were finally determined to be high-risk SSA/Ps, but the remaining 98 cases (74%) were classified into low-risk SSA/Ps. Notably, the high-risk subgroup was characterized by a specific age group and lesion location (Table 1, Fig. 3). CIMP-high and MLH1 methylation were found only in individuals aged 50 years or more (Fig. 3A, Supplementary Table S1, Supplementary Fig. S1H). Moreover, high-risk SSA/Ps were located exclusively in the proximal colon, including cecum, ascending colon, hepatic flexure, and transverse colon (Fig. 3B), whereas low-risk SSA/Ps were distributed throughout the whole colorectum, although they were also enriched in the proximal colon (Fig. 3C). Collectively, the probability of high-risk SSA/Ps could be restricted to proximal colon-located SSA/Ps found in 50 or more-aged adults (Fig. 3D).

Differential clinicopathologic characteristics between high-risk and low-risk subgroups of the 132 non-dysplastic SSA/Ps are summarized in Table 1. In detail, high-risk SSA/Ps were significantly associated with older age (≥ 50 years old, 100%; p = .003), males (82%; p = .015), proximal colonic location (100%; p = .001), larger histologically measured lesion size (> 5 mm, 100%; p = .004), and higher number of typical base-dilated serrated crypts (≥ 3 typical crypts, 94%; p = .024). The mean histologically measured lesion size of high-risk SSA/Ps was significantly higher than that of low-risk SSA/Ps (10.3 mm vs 8.4 mm; p = .034) (Fig. 4A). High-risk SSA/Ps were found only in > 5-mm-sized SSA/Ps, whereas a substantial number of low-risk SSA/Ps were 1- to 5-mm-sized lesions (Fig. 4B). The mean number of typical base-dilated crypts for high-risk lesions was also significantly higher than that of low-risk lesions (18.8 vs 10.7, p = .031) (Fig. 4C). However, the distribution of number of typical crypts between high-risk and low-risk SSA/Ps was similar, except for a few high-risk lesions with a very high number of typical crypts (Fig. 4D).

Lastly, high-risk SSA/Ps were not significantly correlated with multiplicity of serrated lesions (Table 1, Supplementary Fig. S3), largest diameter of typical base-dilated crypts (Table 1, Supplementary Fig. S4), or the average LINE-1 methylation level (Table 1, Supplementary Fig. S5).

**DISCUSSION**

Until recently, number (many), size (large), anatomic site (right-sided colon), and morphologic dysplasia of SSA/Ps have been regarded as the potential risk factors for the progression of SSA/Ps into carcinomas.6,10 However, among these potential risk factors, only morphologic dysplasia is based on strong molecular and pathologic evidence, such as high incidences of MLH1 deficiency, CIMP-high, and accompanying carcinomatous component in SSA/Ps with dysplasia.1,14,15 The other potential risk factors for malignant change of SSA/Ps, including number, size, and...
location of SSA/Ps, have been suggested by experts, based mainly on less-than-robust, inconclusive clinicopathologic data. 

Moreover, the ‘cutoff’ values for the number and size of SSA/Ps for the prediction of high-risk lesions have not been clearly established. Therefore, there is a strong need to precisely identify clinicopathologic risk factors for carcinomatous change in SSA/Ps, especially in SSA/Ps without dysplasia. As no official grading or risk-stratifying system exists for non-dysplastic SSA/Ps, investigating the clinicopathologic factors significantly associated with a high-risk subgroup of non-dysplastic SSA/Ps would be a worthwhile study for clinical and pathologic practices.

To define a high-risk subgroup of non-dysplastic SSA/Ps, we used molecular criteria (CIMP-high and/or MLH1 promoter methylation), because the accumulation of CpG island methylation is known as a molecular hallmark of progression of SSA/Ps (Figs. 2B, 5). 

Based on this aspect, both CIMP-high and MLH1 methylation in SSA/Ps indicate lesions that are epigenetically high-potential to develop into carcinoma. CIMP-high is known to be tightly associated with MLH1 methylation in CRCs, and MLH1 has been used as one of the essential markers for determination of CIMP-high status in CRCs. MLH1 deficiency via the methylation of its gene promoter can induce high microsatellite instability (MSI-high) and subsequent genome-wide hypermutated status in the tumor cells of premalignant lesions such as SSA/Ps, and the hypermutated premalignant lesions can rapidly progress into carcinomas.

In our present study, a total of 132 non-dysplastic SSA/Ps were classified into 34 high-risk and 98 low-risk lesions based on the CpG island methylation criteria (CIMP-high and/or MLH1 methylation). We performed statistical analyses using various clinicopathologic factors and found three major factors for potential use as predictive factors for high-risk lesions of non-dysplastic SSA/Ps.
Table 1. Differential clinicopathologic and molecular features between CpG island methylation-based risk subgroups of non-dysplastic SSA/Ps (n=132)

<table>
<thead>
<tr>
<th>Variable</th>
<th>High-risk SSA/Ps</th>
<th>Low-risk SSA/Ps</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Older (≥ 50)</td>
<td>34 (100)</td>
<td>77 (79)</td>
<td>.003</td>
</tr>
<tr>
<td>Younger (&lt;50)</td>
<td>0</td>
<td>21 (21)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>28 (82)</td>
<td>58 (59)</td>
<td>.015</td>
</tr>
<tr>
<td>Female</td>
<td>6 (18)</td>
<td>40 (41)</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal colon</td>
<td>34 (100)</td>
<td>73 (74)</td>
<td>.001</td>
</tr>
<tr>
<td>Distal colorectum</td>
<td>0</td>
<td>25 (26)</td>
<td></td>
</tr>
<tr>
<td>Lesion size (endoscopically-measured) (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larger (≥5)</td>
<td>34 (100)</td>
<td>91 (93)</td>
<td>.19</td>
</tr>
<tr>
<td>Smaller (&lt;5)</td>
<td>0</td>
<td>7 (7)</td>
<td></td>
</tr>
<tr>
<td>Lesion size (histologically-measured) (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larger (≥5)</td>
<td>34 (100)</td>
<td>80 (82)</td>
<td>.004</td>
</tr>
<tr>
<td>Smaller (&lt;5)</td>
<td>0</td>
<td>18 (18)</td>
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<tr>
<td>Multiplicity (total number of synchronous serrated lesions)</td>
<td>5 (15)</td>
<td>24 (24)</td>
<td>.235</td>
</tr>
<tr>
<td>More (≥3 serrated lesions)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Less (&lt;3 serrated lesions)</td>
<td>29 (85)</td>
<td>74 (76)</td>
<td></td>
</tr>
<tr>
<td>Number of typical base-dilated serrated crypts</td>
<td>32 (94)</td>
<td>75 (77)</td>
<td>.024</td>
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<tr>
<td>More (≥3 typical crypts)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less (&lt;3 typical crypts)</td>
<td>2 (6)</td>
<td>23 (23)</td>
<td></td>
</tr>
<tr>
<td>Largest diameter of typical base-dilated serrated crypts (μm)</td>
<td>19 (56)</td>
<td>37 (38)</td>
<td>.065</td>
</tr>
<tr>
<td>Larger (≥251)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smaller (&lt;251)</td>
<td>15 (44)</td>
<td>61 (62)</td>
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<tr>
<td>MLH1 promoter methylation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Methylated</td>
<td>8 (24)</td>
<td>0</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>26 (76)</td>
<td>98 (100)</td>
<td></td>
</tr>
<tr>
<td>CACNA1G promoter methylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>31 (91)</td>
<td>21 (21)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>3 (9)</td>
<td>77 (79)</td>
<td></td>
</tr>
<tr>
<td>SOCS1 promoter methylation</td>
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<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>12 (35)</td>
<td>7 (7)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>22 (65)</td>
<td>91 (93)</td>
<td></td>
</tr>
<tr>
<td>CRABP1 promoter methylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>34 (100)</td>
<td>47 (48)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>0</td>
<td>51 (52)</td>
<td></td>
</tr>
<tr>
<td>RUNX3 promoter methylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>24 (71)</td>
<td>12 (12)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>10 (29)</td>
<td>86 (88)</td>
<td></td>
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<tr>
<td>IGF2 promoter methylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>28 (82)</td>
<td>13 (13)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>6 (18)</td>
<td>85 (87)</td>
<td></td>
</tr>
<tr>
<td>CDKN2A promoter methylation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Methylated</td>
<td>28 (82)</td>
<td>27 (28)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>6 (18)</td>
<td>71 (72)</td>
<td></td>
</tr>
<tr>
<td>NEUROG1 promoter methylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>33 (97)</td>
<td>45 (46)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>1 (3)</td>
<td>53 (54)</td>
<td></td>
</tr>
<tr>
<td>LINE-1 methylation level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relatively hypermethylated (average level ≥78.81)</td>
<td>21 (62)</td>
<td>48 (49)</td>
<td>.198</td>
</tr>
<tr>
<td>Relatively hypomethylated (average level &lt;78.81)</td>
<td>13 (38)</td>
<td>50 (51)</td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as number (%).
SSA/P, sessile serrated adenoma/polyp; LINE-1, long interspersed nucleotide element-1.
SSA/Ps: patient age, lesion location, and histologically measured lesion size (Fig. 5).

First, age is a significant factor for molecularly defined high-risk SSA/Ps. According to our results, the high-risk SSA/Ps were found exclusively in patients aged 50 years or more (Table 1, Fig. 3A, D). This finding suggests that most of the SSA/Ps found in individuals under the age of 50 may be molecularly benign lesions, with a low risk of progression into malignancy. In fact, the present as well as previous studies suggest that age is important for understanding the characteristics of CpG island methylation in SSA/Ps. Liu et al.\textsuperscript{23} recently published data highlighting the importance of age as a risk factor for CIMP-high and malignant progression in SSA/Ps.\textsuperscript{24} According to these studies, SSA/Ps in young patients rarely demonstrate CIMP-positive (CIMP-high) status, indicating a limited risk of malignant change in SSA/Ps of young patients.\textsuperscript{23,24} These results are very similar to our data. If consistent data are accumulated in future studies as well, it would strengthen the case for using age as an important risk-predictive factor in surveillance and diagnostic guidelines for SSA/Ps.

Our study also revealed that the lesion location could be a sig-
significant factor for the high-risk subgroup of non-dysplastic SSA/Ps. All 34 high-risk SSA/Ps were located exclusively in the proximal colon, including cecum, ascending colon, hepatic flexure, and transverse colon (Table 1, Fig. 3B, D). This finding is not surprising, as previous studies have also suggested that the proximal colonic location is important for malignancy risk of SSA/Ps. The recent study by Liu et al. found that CIMP-high was significantly correlated with proximal location in SSA/Ps, although a small number of distal-located SSA/Ps were also determined to be CIMP-high lesions. Through our results, the importance of lesion location in the prediction of malignant potential of SSA/Ps has been further confirmed.

In addition to patient age and lesion location, histologically measured lesion size was also a significant factor for high-risk SSA/Ps without dysplasia. All 34 high-risk SSA/Ps measured at least 6 mm in size, and the differences of histologically measured sizes between high-risk and low-risk SSA/Ps were statistically significant (Table 1, Fig. 4A, B). Notably, lesion size measured by endoscopy was not a statistically significant factor for the discrimination of high-risk lesions from low-risk SSA/Ps (Table 1, Supplementary Fig. S6). Although, similar to histologically measured lesion size, the endoscopically measured lesion size of all 34 high-risk SSA/Ps was at least 5 mm or more, when measured by endoscopy (Table 1), which likely led to the lack of statistical significance. These findings collectively indicate that histologically measured lesion size may be more valuable and crucial for the risk evaluation of SSA/Ps than endoscopically measured size. The superior value of lesion size measured by histomorphologic methods in the prediction of high-risk SSA/Ps should be validated by multiple independent studies in the future.

As mentioned earlier, although the multiplicity of serrated lesions has been regarded as a potential risk factor for malignant progression of SSA/Ps, the increased number of synchronous serrated lesions was not a significant factor for high-risk non-dysplastic SSA/Ps in our data (Table 1, Supplementary Fig. S3). In fact, because our study excluded cases satisfying diagnostic criteria for serrated polyposis, the expected high-risk lesions in serrated polyposis might be substantially removed from our study samples. As SSA/Ps in serrated polyposis might be clinicopathologically and molecularly different from sporadic SSA/Ps, we decided to focus on sporadically arising SSA/Ps in this study. Therefore, the value of multiplicity of serrated lesions in the prediction of high-risk SSA/Ps should not be underestimated based on the results of our study alone. Nevertheless, our data tend to suggest that multiplicity of serrated lesions may be less valuable than age, location, or size, for prediction of high-risk lesions among sporadic SSA/Ps.

In addition to the identification of clinicopathologic factors associated with the high-risk subgroup of non-dysplastic SSA/Ps, the elucidation of developmental pattern of CpG island methylation in SSA/Ps is another important finding from our study. Based on concurrently methylated marker-dependent and age-dependent frequencies of methylation of each CIMP marker in 132 non-dysplastic SSA/Ps (Fig. 2A, Supplementary Fig. S1), we suggested a sequential model of methylation of CIMP markers in serrated neoplasia pathway (Fig. 2B). Early-methylated markers such as CRABP1 and NEUROG1 could be methylated in SSA/Ps of young patients, whereas late-methylated markers such as SOCS1 and MLH1 could get methylated only in SSA/Ps of patients over the age of 50 (Supplementary Fig. S1). These results

---

Table 1. Clinicopathologic risk factors

<table>
<thead>
<tr>
<th>Clinicopathologic risk factors</th>
<th>Age: ≥ 50</th>
<th>Location: proximal colon</th>
<th>Histologic size: &gt; 5 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-risk (CIMP-low/neg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-risk (CIMP-high)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

Fig. 5. A graphical summary of this study. Age, location, and size are potential predictive factors for a high-risk subgroup of non-dysplastic sessile serrated adenomas/polyps (SSA/Ps). CIMP, CpG island methylator phenotype; MSI, microsatellite instability.
indicate that the accumulation of CpG island methylation during the progression of SSA/Ps is not a stochastic process, but rather an age-dependent, directed process. Our finding is valuable in understanding the characteristics of epigenetic alterations in colorectal carcinogenesis, because currently, the detailed pattern of CIMP development in serrated neoplasia pathway is poorly understood.

Promoter CpG island methylation in MLH1 gene is a critical step for the development of MSI-high/CIMP-high CRCs from SSA/Ps. When MLH1 is methylated and downregulated in an SSA/P without dysplasia, MSI-high and hypermutated phenotype can be induced in the genome of the SSA/P, and the non-dysplastic SSA/P can rapidly progress into dysplasia and carcinoma. Based on our results, it can be inferred that the following two conditions should be satisfied for the MLH1 gene to be methylated in non-dysplastic SSA/Ps: (1) other CIMP markers sufficiently methylated in an SSA/P (generally three or more methylated markers) (Fig. 2A), and (2) old age of the patient (generally ≥ 50 years old) (Supplementary Fig. S1). We also found that both complete loss of MLH1 expression and morphologic dysplasia may occur in SSA/Ps, when MLH1 promoter CpG islands are sufficiently methylated (Supplementary Table S1). MLH1 methylations in non-dysplastic SSA/Ps are mainly subrepressive alterations, because all MLH1-methylated non-dysplastic SSA/Ps demonstrated relatively low PMR values and did not show a complete loss of MLH1 expression (Supplementary Table S1, Supplementary Fig. S2). Thus, MLH1 IHC may not be useful for the screening of high-risk lesions among non-dysplastic SSA/Ps.

In conclusion, CpG island methylation may be an age-dependent stepwise process in the colorectal serrated neoplasia pathway. Both CIMP-high and MLH1 methylation are late-step alterations during the progression of SSA/Ps and are unlikely to occur in SSA/Ps of young patients. Proximal colon-located, > 5 mm-sized SSA/Ps found in individuals aged ≥ 50 should be considered as potential high-risk lesions, regardless of morphologic dysplasia or lesion multiplicity (Fig. 5).

Electronic Supplementary Material
Supplementary materials are available at Journal of Pathology and Translational Medicine (https://jpatholtm.org).

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

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**Supplementary Table S1.** Comparison of the methylation values of CIMP markers and MLH1 IHC results between 8 MLH1-methylated non-dysplastic SSA/Ps (case ID, SSA-xx; included in the study samples) and 2 MLH1-methylated dysplastic SSA/Ps (case ID, SSA-D-xx; not included in the study samples)

<table>
<thead>
<tr>
<th>Case ID</th>
<th>Age (yr)</th>
<th>PMR value of CIMP marker</th>
<th>MLH1 IHC</th>
<th>Morphologic dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CACNA1G</td>
<td>CRABP1</td>
<td>IGF2</td>
</tr>
<tr>
<td>SSA-04</td>
<td>78</td>
<td>46.6</td>
<td>57.4</td>
<td>38.9</td>
</tr>
<tr>
<td>SSA-10</td>
<td>50</td>
<td>25.6</td>
<td>20.3</td>
<td>26.1</td>
</tr>
<tr>
<td>SSA-23</td>
<td>60</td>
<td>1.2</td>
<td>12.1</td>
<td>4.3</td>
</tr>
<tr>
<td>SSA-38</td>
<td>56</td>
<td>0</td>
<td>37.2</td>
<td>27.2</td>
</tr>
<tr>
<td>SSA-55</td>
<td>71</td>
<td>15.2</td>
<td>10.1</td>
<td>4</td>
</tr>
<tr>
<td>SSA-66</td>
<td>63</td>
<td>10.6</td>
<td>27.4</td>
<td>0</td>
</tr>
<tr>
<td>SSA-92</td>
<td>74</td>
<td>46.2</td>
<td>51.4</td>
<td>38.7</td>
</tr>
<tr>
<td>SSA-107</td>
<td>53</td>
<td>74.3</td>
<td>86.1</td>
<td>0.9</td>
</tr>
<tr>
<td>SSA-D-01</td>
<td>65</td>
<td>48.71</td>
<td>41.24</td>
<td>12.71</td>
</tr>
<tr>
<td>SSA-D-04</td>
<td>55</td>
<td>15.1</td>
<td>13.21</td>
<td>17.41</td>
</tr>
</tbody>
</table>

CIMP, CpG island methylator phenotype; IHC, immunohistochemistry; SSA/Ps, sessile serrated adenomas/polyps; PMR, percentage of methylated reference.
Supplementary Fig. S1. Age-dependency of the methylation of specific CpG island methylator phenotype (CIMP) markers in sessile serrated adenomas/polyps (SSA/Ps). Blue bars indicate the percentage of specific CIMP marker methylation-positive cases in the SSA/Ps of each age subgroup. Note the early occurrence of methylation of CRABP1 and NEUROG1 in the SSA/Ps of the younger age group (< 40 years old) (A, B), whereas the methylation of SOCS1 and MLH1 occurred only in the SSA/Ps of the older age group (≥ 50 years old) (G, H). Methylation of CDKN2A (p16), CACNA1G, IGF2, and RUNX3 occurred only in SSA/Ps of ≥ 40 years groups (C–F).
Supplementary Fig. S2. MLH1 immunohistochemistry results for MLH1-methylated sessile serrated adenomas/polyps (SSA/Ps). (A) A case of MLH1-methylated non-dysplastic SSA/P, showing intact MLH1 expression. (B, C) A case of MLH1-methylated non-dysplastic SSA/P, showing partial loss of MLH1 expression. Note the MLH1-negative crypts (in red-lined circles) in the background of MLH1-positive crypts. (D) A case of MLH1-methylated dysplastic SSA/P, showing complete loss of MLH1 expression.
Supplementary Fig. S3. Frequencies of high-risk versus low-risk subgroups of 132 non-dysplastic sessile serrated adenomas/polyps (SSA/Ps), according to the multiplicity of serrated lesions (total number of synchronous serrated lesions).
Supplementary Fig. S4. A comparison of the largest diameters of typical base-dilated serrated crypts between high-risk and low-risk subgroups of 132 non-dysplastic sessile serrated adenomas/polyps (SSA/Ps).
Supplementary Fig. S5. A comparison of the average LINE-1 methylation levels between high-risk and low-risk subgroups of 132 non-dysplastic sessile serrated adenomas/polyps (SSA/Ps).
Supplementary Fig. S6. A comparison of endoscopically measured lesion sizes between high-risk and low-risk subgroups of 132 non-dysplastic sessile serrated adenomas/polyps (SSA/Ps).
Serous Adenocarcinoma of Fallopian Tubes: Histological and Immunohistochemical Aspects

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Primary cancer of the fallopian tubes (PCFT) has the lowest incidence of tumors of the female reproductive organs, but its mortality is quite high.1 It was first described in a small number of patients as sporadic observations. The cause, frequency, diagnosis, prevention, and treatment of PCFT are insufficiently illuminated in the modern medical literature. The incidence of malignant tumors of the fallopian tubes (FT) ranges from 0.14% to 1.8% among all malignant tumors of the female reproductive system.2,3 While it is difficult to identify the initial tumor process of tubo-ovarian tumors, FT malignant tumors are often regarded as a type of ovarian cancer. Consequently, these tumors are much more frequent in gynecologic practice.4-6 This causes significant discrepancies regarding reports of PCFT morbidity, which undoubtedly depends on the quality of morphologic diagnosis.

Practically, FT carcinomas are found more frequently in oncogynecology. Moreover, they serve as the primary source for serous and mucinous tumors of the ovaries and peritoneum.7-11 Increased oncologic awareness and improved diagnostic methods have led to a relative increase in the PCFT detection rate in recent years.12 The main prognostically important criteria remain tumor clinical stage and their degree of malignancy.13,14 The rarity of PCFT has made it impossible to identify significant predictive factors thus far. Identification of tumor markers in PCFT tissue may provide important diagnostic and prognostic information about the biological properties of this neoplasm.

Information about tumor hormonal status (estrogen receptor [ER] and progesterone receptor [PR]), proliferative potential (Ki-67), apoptosis (p53, Bcl-2), and pro-angiogenic (vascular endothelial growth factor) quality of serous tumors were studied in comparison with clinical and morphological characteristics.

Results: ER and PR expression is accompanied by low grade neoplasia, early clinical disease stage, and absence of lymphogenic metastasis (p < .001). HER2/neu expression is not typical for primary cancer of the fallopian tubes. Ki-67 expression is characterized by an inverse correlation with ER and PR (p < .05) and is associated with lymphogenic metastasis (p < .01). p53+ status correlates with high grade malignancy, tumor progression, metastasis, negative ER/PR (p < .001), and negative Bcl-2 status (p < .05). Positive Bcl-2 status is positively correlated with ER and PR expression and low grade malignancy.

Conclusions: Complex morphologic (histological and immunohistochemical) study of postoperative material allows estimation of the degree of malignancy and tumor spread to enable appropriate treatment for each case.

Key Words: Carcinoma, serous; Neoplasms; Fallopian tubes; Estrogen receptor; Progesterone receptor; Ki-67; HER2/neu; p53; Bcl-2; Vascular endothelial growth factor

Background: Although primary cancer of the fallopian tubes is a relatively rare type of tumor in female reproductive organs, its mortality is quite high. It is important to identify molecular and biological markers of this malignancy that determine its specific phenotype.

Methods: The study was carried out on samples received from 71 female patients with primary cancer of the fallopian tubes. The main molecular and biological properties, including hormone status (estrogen receptor [ER], progesterone receptor [PR]), human epidermal growth factor receptor (HER2/neu expression, proliferative potential (Ki-67), apoptosis (p53, Bcl-2), and pro-angiogenic (vascular endothelial growth factor) quality of serous tumors were studied in comparison with clinical and morphological characteristics.

Materials and Methods

The material was collected from Sunny Regional Pathology...
Clinical Center and Sumy Regional Clinical Oncology Center. Written informed consent to investigate tissue was obtained from patients. The Bioethics Commission of the Medical Institute of Sumy State University approved the experimental protocol (No. 8 from 29.04.2016).

The research was performed on surgical material obtained from 71 female patients with PCFT. Pathologic findings were identified using SEE-FIM protocols of FT tissue examination. Determination of the primacy of FT lesions was based on the following criteria: main tumor focus localized in the FT with spread from the endosalpinx for ovary and/or uterus involvement; FT contained more neoplastic mass tissue than other sites; border between normal and affected (neoplastic) epithelium of the tube was visualized; no malignant neoplasms of other localizations.

Hematoxylin-eosin stained tissue sections of 5-μm thickness allowed determination of the histogenesis, degree of tumor malignancy, depth of invasion, spread into pelvic organs, and presence of metastases in regional lymph nodes.

For immunohistochemical study, 4-μm sections were made from paraffin blocks, subjected to standard deparaffinization, dehydrated in xylene and ethanol of graded concentrations, and mounted on 3-aminopropyltriethoxysilane-coated slides. Antigen retrieval was carried out in a water-bath at 97°C–98°C. Antigen-antibody reaction was visualized using the Ultra Vision Quanto Detection System HRP DAB Chromogen (Thermo Fisher Scientific, Waltham, MA, USA), which included blocking endogenous peroxidase activity, blocking nonspecific background staining using the Ultra V block, and enhancing the reaction by Primary Antibody Amplifier Quant. The final visualization was carried out with diaminobenzidine with additional nuclei counterstaining with Modified Mayer’s Hematoxylin (Thermo Fisher Scientific).

To exclude influences of tumor histogenesis on the immunophenotype of cancer cells, the presence of serous adenocarcinoma of the FT (SAcFT) was a criterion for inclusion in the studied group to investigate its molecular-biological markers. All other types (mucinous, squamous, etc.) of PCFT were excluded. While there were few excluded cases, they may have influenced the obtained results. The antibody panel specified in Table 1 was used for this research (Thermo Fisher Scientific).

Obtained specimens were examined using a microscope with digital camera Zeiss AxioCam ERs 5s and ZEN 2 (blue edition) software (Zeiss, Jena, Germany).

Data processing was carried out using Microsoft Excel 2010 with the application Attestar 12.0.5. Estimation of probable differences between comparable indicators was carried out using the Student’s t-test. Detection and evaluation of the links between indicators was carried out using the nonparametric Spearman correlation coefficient (r). A p-value of 0.05 and 95% confidence interval was considered statistically significant.

RESULTS

Table 1. Antibody panel used for immunohistochemical research

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunized animal</th>
<th>Clone</th>
<th>Dilution</th>
<th>Pattern</th>
<th>Evaluation features</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER</td>
<td>Rabbit</td>
<td>SP1</td>
<td>1:200</td>
<td>Nucleus</td>
<td>According to the recommendations of D.C. Allred and taking into account the proportion of colored nuclei and the intensity of their staining</td>
</tr>
<tr>
<td>PR</td>
<td>Rabbit</td>
<td>YR85</td>
<td>1:150</td>
<td>Nucleus</td>
<td>In points (from 0 to 3), according to the manufacturer’s recommendations and taking into account the completeness and intensity of membrane staining</td>
</tr>
<tr>
<td>HER2/neu</td>
<td>Rabbit</td>
<td>SP3</td>
<td>1:100</td>
<td>Membrane</td>
<td>Weak reaction (10–25% of positively stained tumor cells), moderate positive reaction (25%–60%) and strong positive reaction (&gt; 50%)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Rabbit</td>
<td>SP6</td>
<td>1:100</td>
<td>Nucleus</td>
<td>Weak expression (1+): weak cytoplasmic staining of more than 10% of tumor cells</td>
</tr>
<tr>
<td>p53</td>
<td>Mouse</td>
<td>SP5</td>
<td>1:100</td>
<td>Nucleus</td>
<td>Moderate (2+): staining of average intensity more than 10%</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Mouse</td>
<td>100/D5</td>
<td>1:100</td>
<td>Cytoplasm</td>
<td>Strong (3+): intense staining of more than 10% of tumor cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Rabbit</td>
<td>Polyclon</td>
<td>1:200</td>
<td>Cytoplasm and membrane</td>
<td>0 points: absence of cytoplasmic expression</td>
</tr>
</tbody>
</table>

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor; VEGF, vascular endothelial growth factor.
that PCFT accounted for 1.13% of malignant neoplasms of the female reproductive system and 4.36% of malignant neoplasms of the uterine appendages (peak incidence was between 50 and 69 years). FT carcinomas were predominantly localized on one side, and bilateral involvement of tubes was detected in only 16.9% of cases. The tumor process involved the ampulla segment more often (87.3%) than the isthmus (7.1%) or infundibulum (5.6%). In most cases, FT neoplasias (60.6%) were identified at stages I–II (Table 2).

Neoplastic spread outside the FT was found in 66.2% of women during diagnosis. Metastatic tumor dissemination into the omentum, peritoneum, ovaries, and regional lymph nodes did not depend on the size of the primary focus in the FT. Nevertheless, metastases in iliac and lumbar lymph nodes were detected in 26.8% of cases.

**Table 2. Division of PCFT cases according to FIGO classification**

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of patients with PCFT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>24 (33.8)</td>
</tr>
<tr>
<td>IA–T1N0M0</td>
<td>5 (7.0)</td>
</tr>
<tr>
<td>IB–T1bN0M0</td>
<td>8 (11.3)</td>
</tr>
<tr>
<td>IC–T1cN0M0</td>
<td>11 (15.5)</td>
</tr>
<tr>
<td>II</td>
<td>19 (26.8)</td>
</tr>
<tr>
<td>IIA–T2aN0M0</td>
<td>10 (14.1)</td>
</tr>
<tr>
<td>IIB–T2bN0M0</td>
<td>3 (4.2)</td>
</tr>
<tr>
<td>IIC–T2cN0M0</td>
<td>6 (8.5)</td>
</tr>
<tr>
<td>III</td>
<td>28 (39.4)</td>
</tr>
<tr>
<td>IIIA–T3aN0M0</td>
<td>2 (2.8)</td>
</tr>
<tr>
<td>IIIB–T3bN0M0</td>
<td>5 (7.0)</td>
</tr>
<tr>
<td>IIIC–T3cN0M0</td>
<td>2 (2.8)</td>
</tr>
<tr>
<td>IIIIC–T1-3N1M0</td>
<td>19 (26.8)</td>
</tr>
<tr>
<td>IV–T1-3N0-1M1</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>71 (100)</td>
</tr>
</tbody>
</table>

PCFT, primary cancer of the fallopian tubes; FIGO, International Federation of Gynecology and Obstetrics.

Our histologic study showed that the overwhelming majority of malignant neoplasms of the FT were SAcFT (93.0% of cases) (Fig. 1). Mucinous (2.8%), clear cell (2.8%), and squamous cell carcinomas (1.4%) were quite rare.

A total of 88.4% of SAcFT cases showed a high degree of malignancy (low [G3] and moderately differentiated [G2]). Their metastases were more often present in regional lymph nodes (28.2%), omentum, peritoneum (11.3%), and ovaries (5.6%).

Immunohistochemical study showed variable expression of ER and PR (Fig. 2A, B): ER+PR+, 62.1%; ER+PR−, 21.2%. Strong expression of ER and PR in SAcFT correlated with well-differentiated tumors (low grade cancer) and initial clinical stages of disease (p < .001). The presence of metastases in lymph nodes was accompanied by significantly lower expression of ER (p < .001) and PR (p < .001) in cancer cells compared to cases without metastases (p < .05).

Human epidermal growth factor receptor (HER2)/neu overexpression was not typical for SAcFT (80.9% of tumors were receptor-negative). Nevertheless, most neoplastic FT cells (55.0 ± 12.2%) expressed Ki-67 receptors (Fig. 2C, D). Table 3 shows the division of patients by Ki-67 expression level.

Tumor cell proliferation was characterized by an inverse relationship with steroid hormone expression (p < .05). In groups with positive receptor status (ER+PR+), Ki-67 overexpression (more than 60%) was detected in only 46.3% of cases. Conversely, tumors with completely negative ER and PR or with only ER positivity had significantly higher proliferative activity (63.4% and 71.4%, respectively). There were no differences in Ki-67 expression, clinical tumor stage, or degree of differentiation (p > .05).

In 13 cases (19.7%), a negative reaction was detected for the pro-apoptotic protein p53 (Fig. 2F). Fifty-three cases of SAcFT (80.3%) were p53-positive. Among them, nine cases (13.6%) were weakly positive, 13 cases (19.7%) were moderately posi-

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**Fig. 1. Serous adenocarcinoma of the fallopian tube. (A) G1 (low degree of malignancy). (B) G2 (high degree of malignancy). (C) G3 (high degree of malignancy).**

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http://jpatholtm.org/  
https://doi.org/10.4132/jptm.2019.03.21
Fig. 2. Serous adenocarcinoma of the fallopian tube. Immunohistochemical study of estrogen receptors (A, B), Ki-67 (C, D), p53 (E, F), and vascular endothelial growth factor (G, H) expression.
Table 3. Division of female patients by level of Ki-67 expression in SAcFT tissue

<table>
<thead>
<tr>
<th>Average index of proliferation (%)</th>
<th>No. of female patients with SAcFT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0–30</td>
<td>12 (18.2)</td>
</tr>
<tr>
<td>30–60</td>
<td>18 (27.3)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>36 (54.5)</td>
</tr>
</tbody>
</table>

SAcFT, serous adenocarcinoma of the fallopian tubes.

Table 4. Results of p53 expression study in SAcFT depending on prevalence, presence of lymphogenous metastases, and degree of malignancy

<table>
<thead>
<tr>
<th>Evaluation’s criteria</th>
<th>Average No. of p53 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease stage according to FIGO classification</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>10.5 ± 2.2</td>
</tr>
<tr>
<td>II</td>
<td>46.8 ± 5.2</td>
</tr>
<tr>
<td>III</td>
<td>71.7 ± 3.9</td>
</tr>
<tr>
<td>Presence of lesion in regional lymph nodes</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>29.7 ± 3.6</td>
</tr>
<tr>
<td>N1</td>
<td>80.6 ± 2.7</td>
</tr>
<tr>
<td>Degree of tumor differentiation</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>5.6 ± 2.4</td>
</tr>
<tr>
<td>G2</td>
<td>35.6 ± 6.2</td>
</tr>
<tr>
<td>G3</td>
<td>62.2 ± 4.2</td>
</tr>
</tbody>
</table>

SAcFT, serous adenocarcinoma of the fallopian tubes; FIGO, International Federation of Gynecology and Obstetrics.

cells and vascular endothelium. Most patients had moderate or strong cytoplasmic signals in endothelial cells (87.9%) and in more than 70% of tumor cells located diffusely in all fields of view (Fig. 2G, H). Only six cases (9.1%) had weak and moderate focal expression of 10%–50%. Focal weak cytoplasmic reaction, which was present in less than 10% of tumor cells and in the endothelium of single vessels, was observed in only two cases (3%).

The angiogenic potential of PCFT depends on the degree of differentiation (p < .05) and clinical disease stage (p < .05). Moreover, all cases of regional lymph node metastases were accompanied by strong VEGF expression. This result may confirm that metastasis of PCFT depends on angiogenesis. In general, steroid-negative FT tumors were characterized by a higher level of VEGF expression. There was weak correlation between expression of VEGF and p53 (r = 0.25, p < .05). There was no significant correlation between VEGF expression and the presence of Ki-67 and Bcl-2 receptors (p > .05).

**DISCUSSION**

Numerous scientific publications on PCFT include single observations of this disease.15,16 Patients with malignant FT neoplasms are typically older, which may be due to increased amount of secretory cells in older women.17,18 Morphologically, malignant epithelial tumors of the FT can be comprised of carcinomas of all cell types. In our research, the overwhelming majority of malignant neoplasms were SAcFT, which demonstrate a high degree of malignancy and very aggressive behavior.

The presence of steroid hormone receptors in FT tumors is a
substantial indicator for the use of hormonal therapy. Conversely, steroid-negative tumors are contraindicated for hormone therapy. The medical literature includes sporadic reports on the use of hormonal therapy combined with cytostatics for treatment of FT neoplasia. Increased anaplasia and significant tumor spread are associated with decreased steroid-positive cells and an increased proportion of steroid-negative FT neoplasms. This can be explained by functional and phenotypic simplification of tumor cells with decreased ER and PR expression associated with tumor progression (increased tumor autonomy degree). Moreover, neoplasias with lymph node metastases expressed significantly less ER and PR than cases without lymph node metastases (p < .001).

Our results demonstrate that the presence of steroid hormone receptors in SAcFT is an important prognostic biologic characteristic of these neoplasias. Reduced or lost expression of both ER and PR in FT tumors is associated with invasion, metastasis, and an adverse disease course.

The results of our research indicate that overexpression of HER2/neu is not typical for PCFT. In contrast to our data, overexpression of HER2/neu in neoplastic FT tissue has been demonstrated by some authors in 25.6%, 31%, and even 89% of SAcFT. According to our results, we cannot recommend testing for this oncoprotein as a prognostic biomarker.

Immunohistochemistry demonstrated a mean Ki-67 of 55.0% ± 12.2%. Most FT tumors have a proliferation index of more than 60% (54.5%). PCFT has high proliferative activity, indicating an extremely aggressive disease course. In the presence of metastases, the level of Ki-67 expression can be considered an independent prognostic marker for N-status in PCFT. This contributes to the allocation of certain women to the high-risk patient group.

The received data suggests that there is no direct dependency between a cell’s steroid status and its ability to divide. This indicates that complex hormonal mechanisms contribute to proliferation. The significant increase in proliferative activity of PR cells may demonstrate the significant effect of progesterone on the ability of cells to divide.

In our study, 80.3% of SAcFT cases were p53-positive. p53 mutations were associated with tumor spread. In contrast to p53, 72.4% of cases expressed the anti-apoptotic Bcl-2 protein in the first stages of the neoplastic process. Blockage of apoptosis likely occurs in one of two tumor growth stages via different mechanisms; in the early stages, Bcl-2 is activated, while in the late stages, mutations in the TP53 gene occur. This results in the loss of functional qualities of the protein that it encodes. Exuberant amounts of mutant p53 and decreased wild-type p53 lead to suppression of the apoptosis blockade, which consequently

Fig. 3. Scheme mutant (mt) of p53 participation in carcinogenesis of primary cancer of the fallopian tubes. DNA damage and appearance of mt p53 proteins results in suppression of blocked apoptosis. Conversely, it also stimulates increased antiapoptotic Bcl-2 proteins, which enhance the inhibitory effect on apoptosis. This is manifested in altered cell cycle regulation. All of these events lead to increased proliferative tumor activity and progression of malignancy.
increases cell proliferation. Cell division becomes uncontrolled and the malignant process actively progresses (Fig. 3).

The p53 + status of SAcFT correlates with a high degree of malignancy, significant tumor spread, metastases, and high proliferation. In other words, p53 + status can act as an independent prognostic factor for PCFT. In addition, our studies show that the Bcl-2 + status of FT tumors correlates with positivity of steroid hormone receptors, low degree of anaplasia, and low proliferation. Bcl-2 overexpression indicates more intense inhibition of apoptosis and should be accompanied by more aggressive tumor behavior. Nonetheless, according to our obtained data, Bcl-2 overexpression is a positive predictor for PCFT. This is likely due to a combination of Bcl-2 and steroid hormone receptor expression since the Bcl-2 gene is estrogen-dependent.26,27

FT tumors are characterized by robust angiogenesis. Sometimes, tumor cells can stimulate angiogenesis (angiogenic activation) by forming and releasing angiogenic factors (e.g., VEGFs) that stimulate endothelial cell proliferation and capillary growth.28,29 In our research, the majority of patients (87.9%) had moderate or strong VEGF cytoplasmic expression in endothelial cells and in more than 70% of tumor cells were located diffusely in all fields of view. Accordingly, constant and intense angiogenesis in PCFT determines their fast growth and has adverse prognostic implications. We have also detected that the angiogenic potential of malignant FT tumors correlates with the degree of differentiation, clinical disease stage, negative ER/PR status, and p53-positive status (p < .05). All cases of metastasis in regional lymph nodes were accompanied by strong VEGF expression.

The results of our complex morphological study of PCFT demonstrate factors that should be considered when determining the strategy for treating patients with malignant FT tumors, including use of antihormonal therapy in cases of steroid positive tumors, adjuvant chemotherapy in cases with proapoptotic protein values, p53 higher than 10%, proliferative activity more than 60%, and use of anti-VEGF therapy in cases with high angiogenic potential. Our study was limited by a lack of data on patient lifestyle, which may be important, as it is in neoplastic processes in other female reproductive organs.10,31

In most cases, PCFT is serous adenocarcinoma. Neoplastic cells have high rates of expression for estrogen receptors (83.3%), progesterone receptors (62.1%), Ki-67 (55.0% ± 12.2%), p53 (80.3%), Bcl-2 (81.8%), and VEGF (87.9%), which influence tumor aggressiveness. The use of complex morphologic (histological and immunohistochemical) study of postoperative material allows assessment of the degree of malignancy and tumor spread. This allows selection of appropriate therapeutic tactics for each individual case.

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

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REFERENCES


Prognostic Significance of CD109 Expression in Patients with Ovarian Epithelial Cancer

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Background: Ovarian epithelial cancer (OEC) is the second-most common gynecologic malignancy.1 However, it is often found in advanced stages, which makes it difficult to treat. OEC is believed to have an insidious onset, with no early symptoms.2 Survival rates for women with advanced disease range from 20%–30%, which are much lower than those for women with early stage disease (70%–90%).3 Despite tumor-debulking surgery and chemotherapy, the 50-year survival rate is as low as 40%.4 Although factors associated with the prognosis of OEC, such as p53 and human epidermal growth factor receptor 2 expression, have been reported,5,6 only a few factors can predict poor outcome in patients with OEC.

CD109 is a glycosylphosphatidylinositol-linked cell surface glycoprotein and a member of the 2-macroglobulin-C3, C4, C5 family of thioester-containing proteins.7 CD109 expression is limited in certain cell types in normal tissues, including myoepithelial cells of the breast, salivary gland, and basal cells of the prostate.8 CD109 protein is a component of the transforming growth factor β1 (TGF-β1) receptor system, which is involved in cell proliferation and differentiation and has both tumor-suppressive and promoting effects during carcinogenesis.9 However, its functions are still unknown. A previous study showed that CD109 expression is elevated in human tumor cell lines and carcinomas.10 CD109 expression is significantly increased in carcinomas of the lung, gallbladder, uterine cervix, and vulva and soft tissue sarcomas.10-14 In addition, CD109 expression in lung squamous cell carcinoma is associated with the tumor stage, and its expression in myxofibrosarcomas is useful to predict recurrence.15-17 Thus, CD109 could be expressed in OEC and
may be a useful predictor of OEC prognosis.

The aim of this study was to evaluate CD109 expression by immunohistochemistry and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using clinical specimens of human OEC; to analyze the correlation between CD109 expression and survival rate, recurrence rate, and chemotherapy response in patients with OEC; and to investigate the prognostic significance of CD109 expression.

**MATERIALS AND METHODS**

**Patients and data**

OEC patients who underwent surgical resection at Pusan National University Hospital from 1998 to 2009 were selected for this study. All patients provided written informed consents and underwent surgical procedures. The biospecimens and data used for this study were provided by the Biobank of Pusan National University Hospital (PNUH), a member of the Korea Biobank Network. All samples derived from the National Biobank of Korea were obtained with the approval of institutional review board. After exclusion of cases with insufficient tissue material and clinical information, a total of 120 cases were enrolled and representative formalin-fixed paraffin tissue blocks were collected. Pathological data including histological type, pathological stage, tumor histological grade, nuclear grade, and mitosis were obtained from the primary pathology report. The histological tumor type was classified according to the World Health Organization criteria. Histological grades were classified as well-, moderately, and poorly differentiated according to the Silverberg grading system. The tumors were staged according to the International Federation of Gynecology and Obstetrics (FIGO) staging system (Table 1).

Fresh tissue samples of OEC were obtained by surgical resection and stored in the Biobank of PNUH. For this study, tissue samples from 12 patients who underwent resection were examined. We evaluated 12 patients with serous carcinoma who received chemotherapy. Six patients who did not experience relapse for 18 months after chemotherapy were included in the good-response group, and six patients who experienced recurrence within 6 months of chemotherapy initiation to 8 months of chemotherapy completion were included in the poor-response group (Table 2).

**Immunohistochemistry**

CD109 expression was assessed using CD109 immunohistochemical staining. The slides were then dewaxed in xylene and dehydrated in ethanol. Staining was performed using BondMax-autostainer and other reagents (Leica Microsystems, Berlin, Germany). Deparaaffinization was performed automatically in the autostainer with BondWash solution (Leica Microsystems) at 72°C for 30 minutes. After washing, the slides were incubated overnight at 4°C with a rabbit polyclonal anti-CD109 antibody (dilution 1:50, cat. No., HPA009292, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (dilution 1:100, ProteinTech Group, Inc., Wuhan Sanying Biotechnology, Wuhan, China) for 90 minutes at room temperature. Antibody binding was performed by incubating the slides for 1 minute with a solution of 1 drop of 3,3′-diaminobenzidine (20 ×) per 1.0 mL diamino-benzidine substrate buffer (cat. No. ZLI-9017, OriGene Technologies, Inc.,

<table>
<thead>
<tr>
<th>Table 1. Characteristic of the patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up, median (range, mo)</td>
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<tr>
<td>Age at diagnosis (yr)</td>
</tr>
<tr>
<td>&lt; 50</td>
</tr>
<tr>
<td>≥ 50</td>
</tr>
<tr>
<td>Histologic type</td>
</tr>
<tr>
<td>Serous</td>
</tr>
<tr>
<td>Mucinous</td>
</tr>
<tr>
<td>Endometrioid</td>
</tr>
<tr>
<td>Clear cell</td>
</tr>
<tr>
<td>Undifferentiated</td>
</tr>
<tr>
<td>Histologic grade</td>
</tr>
<tr>
<td>Well differentiated</td>
</tr>
<tr>
<td>Moderate differentiated</td>
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<tr>
<td>Poorly differentiated</td>
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<tr>
<td>Nuclear grade</td>
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<tr>
<td>I</td>
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<tr>
<td>II</td>
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<tr>
<td>III</td>
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<tr>
<td>FIGO stage</td>
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<tr>
<td>I</td>
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<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td>Mitosis (per 10-HPFs)</td>
</tr>
<tr>
<td>1–9</td>
</tr>
<tr>
<td>10–19</td>
</tr>
<tr>
<td>≥ 20</td>
</tr>
<tr>
<td>Overall survival</td>
</tr>
<tr>
<td>Survival</td>
</tr>
<tr>
<td>Death</td>
</tr>
<tr>
<td>Recurrence</td>
</tr>
<tr>
<td>Absent</td>
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<tr>
<td>Present</td>
</tr>
</tbody>
</table>

FIGO, International Federation of Gynecology and Obstetrics; HPF, high-power field.
Beijing, China). The slides were then counterstained with EnVision FLEX hematoxylin (Dako, Agilent Technologies, Inc., Santa Clara, CA, USA) for 1 minute and dehydrated using ethanol and xylene. For negative control, staining was performed without primary antibody.

Assessment of immunohistochemical staining

Slides were evaluated using light microscopy. CD109 expression was detected through cytoplasmic and/or membranous staining of the tumor cells. The CD109 positivity of tumor cells was determined as follows: positive staining, ≥ 10% positive tumor cells and negative staining, < 10% positive tumor cells.

RT-qPCR

The mRNA levels of CD109 in the fresh tissue samples of OEC were measured using RT-qPCR. Total RNA was purified from cells using RNeasy mini prep kits (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1 µg RNA using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA). Differential RNA levels were assessed using Luna Universal qPCR Master Mix (New England Biolabs) and primers for each gene. Quantitative PCR reactions were performed using an ECO Real-Time PCR system (PCRmax, Staffordshire, UK) as follows: 95°C for 30 minutes followed by 40 cycles at 95°C for 10 seconds, 61°C for 30 seconds, and 72°C for 20 seconds. The PCR products were analyzed using ECO ware (PCRmax). All samples were normalized to the signal generated from glyceraldehyde 3-phosphate dehydrogenase using the primers presented in Table 3. The CD109 gene expression was generalized by comparing the relative expression of CD109 with the internal standard glyceraldehyde 3-phosphate dehydrogenase using the 2−ΔΔCT method.19

Statistical analysis

The Pearson’s chi-square test was used to assess statistical significance between CD109 expression and various clinicopathological characteristics. Overall survival and recurrence-free survival were estimated using Kaplan-Meier plots. Multivariate analyses to determine hazard ratios for overall survival and recurrence-free survival were performed using Cox regression analysis. All analyses were performed using SPSS software (IBM, Armonk, NY, USA). A p-value of < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

A series of 120 OEC cases was retrieved for this study. The follow-up period ranged from 1 to 115 months (median, 50 months), and the patient age ranged from 15 to 82 years (median, 50 years). There were 59 cases of serous carcinoma, 22 cases of mucinous carcinoma, nine cases of endometrioid carcinoma, 27 cases of clear cell carcinoma, and three cases of undifferentiated carcinoma. The overall survival rate was 52.5%, and the recurrence rate was 77.5%. Other clinicopathologic parameters are presented in Table 1.
CD109 expression in OEC

In OEC samples, the malignant tumor cells exhibited brownish CD109 staining in the membrane and cytoplasm (Fig. 1). CD109 expression was detected in 63 of the 120 OEC samples (52.5%). CD109 expression differed according to the histological type of tumor (p < .001). There was a statistically significantly increase in the expression of CD109 in serous, endometrioid, and undifferentiated carcinomas (77.4%). CD109 expression was associated with a higher histological tumor grade (p = .012). Twelve of 51 FIGO stage I tumors (23.5%) and 51 of 69 FIGO stage II, III, and IV tumors (73.9%) were positive for CD109 (p < .001). Thirteen of 45 tumors with < 10 mitotic events (28.9%) and 50 of 75 tumors with more than 10 mitotic events (66.7%) were positive for CD109 (p < .001). A high nuclear grade was not associated with CD109 expression (Table 4).

In the above results, CD109 positivity ratio varied according to the histologic type, and the following is the CD109 expression results in serous carcinoma that accounts for the largest percentage of OEC: Six of 13 FIGO stage I tumors (46.2%) and 40 of 46 FIGO stage II, III, and IV tumors (87.0%) were positive for CD109 (p = .004). Histologic grade, high nuclear grade, and high mitotic counts were not associated with CD109 expression (Table 5).

Association between CD109 expression and OEC prognosis

CD109 expression was associated with overall survival (p =

Fig. 1. Immunohistochemical staining of CD109 in ovarian epithelial carcinoma. (A) Positive in serous carcinoma. (B) Positive in mucinous carcinoma. (C) Positive in endometrioid carcinoma. (D) Positive in clear cell carcinoma. (E) Positive in undifferentiated carcinoma. (F) Negative in serous carcinoma.
.020) (Fig. 2), but not recurrence-free survival (p = .290) (Fig. 3). The 5-year survival rate was 42.5% in patients with CD109-positive results and 64.8% in patients with CD109-negative results (p = .020) (Fig. 2). Analysis of the variables using multivariate analysis showed that CD109 expression was not an independent risk factor for overall survival due to its low reliability (hazard ratio [HR], 1.58; p = .160; 95% confidence interval [CI], 0.82 to 3.05) (Table 6). In addition, CD109 expression was not an only risk

Table 4. Correlation between CD109 expression and clinicopathological characteristics in ovarian epithelial carcinomas (n = 120)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative</th>
<th>Positive</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>13 (22.0)</td>
<td>46 (78.0)</td>
<td>.000</td>
</tr>
<tr>
<td>Mucinous</td>
<td>19 (86.4)</td>
<td>3 (13.6)</td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>3 (33.3)</td>
<td>6 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
<td>22 (81.5)</td>
<td>5 (18.5)</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>0 (0)</td>
<td>3 (100)</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
<td>.012</td>
</tr>
<tr>
<td>Well differentiated</td>
<td>19 (67.9)</td>
<td>9 (32.1)</td>
<td></td>
</tr>
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<td>Moderate differentiated</td>
<td>29 (47.5)</td>
<td>32 (52.5)</td>
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<tr>
<td>Poorly differentiated</td>
<td>9 (29.0)</td>
<td>22 (71.0)</td>
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<tr>
<td>Nuclear grade</td>
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<tr>
<td>I</td>
<td>3 (50.0)</td>
<td>3 (50.0)</td>
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<tr>
<td>II</td>
<td>29 (44.6)</td>
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<td>III</td>
<td>25 (51.0)</td>
<td>24 (49.0)</td>
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<tr>
<td>I</td>
<td>39 (76.5)</td>
<td>12 (23.5)</td>
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<tr>
<td>II, III and IV</td>
<td>18 (26.1)</td>
<td>51 (73.9)</td>
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<tr>
<td>Mitoses (per 10HPFs)</td>
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<tr>
<td>1–9</td>
<td>32 (71.1)</td>
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</tr>
<tr>
<td>≥ 10</td>
<td>25 (33.3)</td>
<td>50 (66.7)</td>
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</tr>
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</table>

Table 5. Correlation between CD109 expression and clinicopathological characteristics in serous carcinoma (n = 59)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Negative</th>
<th>Positive</th>
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</thead>
<tbody>
<tr>
<td>Histologic grade</td>
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</tr>
<tr>
<td>Well differentiated</td>
<td>2 (40.0)</td>
<td>3 (60.0)</td>
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<tr>
<td>Moderate differentiated</td>
<td>9 (24.3)</td>
<td>28 (75.7)</td>
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<tr>
<td>Poorly differentiated</td>
<td>2 (11.8)</td>
<td>15 (88.2)</td>
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<td>8 (21.6)</td>
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<td></td>
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<td>III</td>
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<td>16 (76.2)</td>
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<td>I</td>
<td>7 (53.8)</td>
<td>6 (46.2)</td>
<td></td>
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<tr>
<td>II, III and IV</td>
<td>6 (13.0)</td>
<td>40 (87.0)</td>
<td></td>
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<tr>
<td>Mitoses (per 10HPFs)</td>
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<td></td>
<td>.959</td>
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<td>1–9</td>
<td>3 (25.0)</td>
<td>9 (75.0)</td>
<td></td>
</tr>
<tr>
<td>10–19</td>
<td>5 (21.7)</td>
<td>18 (78.3)</td>
<td></td>
</tr>
<tr>
<td>≥ 20</td>
<td>5 (20.8)</td>
<td>19 (79.2)</td>
<td></td>
</tr>
</tbody>
</table>

Negative, less than 10% positive staining of tumor cells; Positive, more than 10% positive staining of tumor cells.

FIGO, Federation of Gynecology and Obstetrics; HPF, high power field.

*Pearson’s chi-squared test.
factor for recurrence-free survival (HR, 2.06; p = .110; 95% CI, 0.83 to 5.09) (Table 7). Therefore, CD109 expression is not an independent prognostic factor but is helpful for predicting the prognosis of patients with OEC.

Immunohistochemical staining was performed in 37 patients with serous carcinoma who received chemotherapy, 21 patients belonged to the good-response group and 16 patients belonged to the poor-response group. The poor-response group had higher rates of CD109 expression than the good-response group (93.8% vs 66.7%, p = .047) (Table 8). Thus, CD109 is useful for predicting the chemotherapeutic effect in patients with OEC, especially serous carcinoma.

**CD109 mRNA levels in OECs**

All 12 fresh OEC samples were examined for CD109 expression by RT-qPCR. The samples were limited to serous carcinoma of OEC. CD109 mRNA was significantly upregulated in the poor-response group compared with the good-response group (p = .001) (Fig. 4), suggesting that high CD109 expression level is associated with chemoresistance in patients with OEC.

**DISCUSSION**

The physiological function of CD109 has not been studied, yet it is highly expressed in various types of malignancies and several normal tissues.\(^8,20\) It has recently been reported that CD109 expression is associated with tumor development and cell proliferation using human oral tumor tissues and cancer cell lines.\(^21\) In addition, CD109 can predict prognosis in tumors such as myxofibrosarcoma.\(^15\) In this study, we evaluated CD109 expression in OEC samples by immunohistochemical stains and RT-qPCR and found that an increased CD109 expression was associated with the prognosis and chemoresistance of OEC.

**CD109 protein is a component of the TGF-β1 receptor system.**\(^9\) Tumor suppression occurs through the TGF-β1–signaling pathway in the early tumor stage; however, in malignantly transformed cells, TGF-β1 is severely dysregulated, resulting in the loss of tumor suppression.\(^8\) CD109 expression is followed by a complex formation with the type I TGF-β1 receptor, which is required for the regulation of TGF-β1 signaling in early tumor cells.\(^22\) Finally, CD109 degrades the TGF-β1 receptor and blocks TGF-β1 signaling, thereby preventing tumor suppression,\(^23\) which was confirmed in a mouse model.\(^24\) In CD109-deficient mice, the TGF-β1 signaling pathway was enhanced and it suppressed skin tumorigenesis.\(^24\)

**TGF-β1 plays an important role in chemoresistance in breast cancer and squamous cell carcinoma.**\(^25,26\) During TGF-β1 expres-
sion, cancer cells proliferate slowly in early tumor stage and lead to chemoresistance because they can be protected against DNA-damaging chemotherapeutic agents. In myxofibrosarcomas, tumors with high CD109 expression level showed decreased expression of TGF-β1 with a good treatment effect. However, tumors with low CD109 expression and high TGF-β1 expression showed chemoresistance. The current case and the other cases mentioned above indicate that there are other underlying mechanisms stronger than the CD109–TGF-β1 signaling pathway. In our study, the relationship between CD109 expression and chemoresistance was identified, but TGF-β1 expression and its role were not confirmed. If both CD109 and TGF-β1 are found to be related to the chemoresistance of OEC, it will be helpful in treating patients. Therefore, further research is needed to confirm these findings in OEC.

CD109 expression has been associated with chemotherapeutic resistance in triple-negative breast cancers. After chemotherapy was administered to patients with CD109-positive triple negative breast cancer, 50% of the patients showed disease progression and none of them showed complete response. The same result was reported when examining the CD109 expression and chemotherapeutic effect in breast cancer and glioblastoma. This may be due to the role of CD109 in the development of endothelial cells and induction of angiogenesis in contrary to the effect of chemotherapy regimen, which blocks tumor neovascularization. In this study, CD109 was expressed at a higher rate in the poor-response group than in the good-response group. In addition, the expression level of CD109 mRNA was 2.88-fold higher in the poor-response group than in the good-response group. However, unlike breast cancer, most OECs are diagnosed at advanced stages, making it difficult to divide the chemotherapy response group into more detailed subgroups.

CD109 is involved in the pathogenesis and prognosis of some tumors. CD109 overexpression is known to induce cell growth in oral squamous cell carcinomas, and CD109-positive oral dysplastic lesions develop into squamous cell carcinoma within 3 years. Although there is no correlation between the CD109 expression and mitosis, CD109 expression was associated with neoplastic cell growth. Recently, a study suggested that CD109 expression is managed by cancer stem-like cells/cancer-initiating cells, which can initiate tumorigenesis and tumor growth in sarcomas. Therefore, in the presence of cancer stem cells, CD109 expression may increase and malignancies or recurrence/metastasis may develop. In our study, CD109 expression was higher in the advanced stage, but OEC precursors such as borderline ovarian tumor is not included in the study. More research that can determine the association of CD109 with neoplastic cell growth in ovary tissue may be helpful in early detection and treatment of patients.

Based on our review, this is the first study on the relationship between CD109 expression and prognosis of OEC. The present study demonstrated that CD109 is highly expressed in OEC tissues. Immunohistochemical analysis of CD109 expression is useful for predicting overall survival but not recurrence-free survival. Regarding the chemotherapeutic effect on serous carcinoma, the poor-response group expressed increased mRNA levels of CD109 as compared to the good-response group. Therefore, identification of CD109 expression may help in predicting the survival and chemotherapeutic effect in patients with serous carcinoma of OEC. Further analysis of other factors related to CD109 is needed for careful management of patients with serous carcinoma of OEC.

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

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Progressive Familial Intrahepatic Cholestasis in Korea: A Clinicopathological Study of Five Patients

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Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of autosomally recessive inherited diseases, manifesting as neonatal cholestasis of hepatocellular origin. PFIC presents as jaundice, pruritus, and failure to thrive in neonates and infants, and usually advances to liver failure.1,2 PFIC was first described in an Amish kindred and called Byler disease.3 Molecular analysis showed distinct genetic mutations, classifying PFIC into three types, with all three characterized by a deficiency of bile transporters in hepatocytes. PFIC1 is characterized by mutations in the ATP8B1 gene, resulting in a deficiency in an aminophospholipid flipase that plays a role in maintaining plasma membrane integrity; PFIC2 is characterized by mutations in the ABCB11 gene (ATP-binding cassette family B, member 11), which encodes a bile salt export pump (BSEP); and PFIC3 is characterized by mutations in the MDR3 (class III multidrug resistance p-glycoprotein) gene, which encodes a flipase required for biliary phosphatidylcholine secretion.4-7 PFIC1 and PFIC2 are both characterized by low serum γ-glutamyl transpeptidase (GGT) concentrations and direct hyperbilirubinemia, findings that are useful for their differential diagnosis from other neonatal cholestatic liver diseases.2 Despite their distinct clinicopathological features, pathologists and pediatricians are unfamiliar with these diseases because they are rare, with an estimated incidence rate of 1:50,000 to 1:100,000 births.2

In Korea, PFIC has not been analyzed in detail and its clinicopathological course after diagnosis has not yet been reported. The present study therefore investigated the clinicopathological features, mutation profiles, and clinical courses of a series of patients at a
single center diagnosed with PFIC.

**MATERIALS AND METHODS**

**Ethical approval**

This study was approved by the Institutional Review Board of Asan Medical Center, which waived the requirement for informed consent (IRB No. 2017-0703).

**Patients and samples**

From 2008 to 2014, five patients were diagnosed with PFIC at Asan Medical Center, Seoul, Republic of Korea. Diagnosis was based on clinical findings, histological and ultrastructural features of liver biopsy or liver transplantation (LT) samples, and mutation profiles. Clinical information, including age at onset, sex, chief complaints, and laboratory data, were retrieved from patients’ electronic medical records. Informed consent for liver biopsy and DNA analysis in all patients was obtained from the patients’ parents. No patients were lost to follow-up and the median follow-up time was 53 months (range, 36 to 102 months).

**Histopathological analysis**

Fifteen specimens from four liver biopsies, eight posttransplant liver biopsies, and three explanted livers were histopathologically analyzed. All specimens were fixed in 10% neutral formalin and processed for hematoxylin-eosin and Masson trichrome staining to analyze histology and the stage of fibrosis. Specimens for electron microscopy were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 48 hours, and postfixed in 1% osmium tetroxide. Ultrathin sections were cut, stained with uranyl acetate, and examined using a transmission electron microscope.

Samples were immunohistochemically stained with antibodies to cytokeratin 19 (1:200, DAKO, Glostrup, Denmark) and BSEP (1:1,000, Sigma Aldrich, St. Louis, MO, USA) to assess bile duct damage and BSEP protein expression, respectively, using a Benchmark XT auto immunostainer (Ventana Medical System, Tucson, AZ, USA) with OptiView DAB IHC Detection kit (Ventana Medical System) according to the manufacturer’s instructions. Histopathological features were carefully reviewed by three pathologists (E.Y., S.A.H., and H.J.K.) specializing in liver diseases, and the absence or presence of ballooning degeneration, giant cell transformation, and ductular reaction, as well as the extent of bile plugs, was determined. Lobular and portal based fibrosis were also analyzed, according to the Korean Society of Pathologists Scoring System. Lobular fibrosis was graded as negative (no perivenular fibrosis), mild (perivenular fibrosis), moderate (perivenular and sinusoidal fibrosis), or severe (diffuse perivenular and sinusoidal fibrosis). Portal fibrosis was graded as no portal fibrosis, periportal fibrosis, septal fibrosis and cirrhosis.

**Genetic test**

Genomic DNA was isolated from peripheral blood leukocytes using Gentra Puregene blood kits (Qiagen, Hilden, Germany). Twenty-seven exons and exon-intron boundaries of *ATP8B1* and 28 exons and exon-intron boundaries of *ABCB11* were amplified by polymerase chain reaction (PCR) using GoTaq Polymerase (Promega, Madison, WI, USA), and the products were directly sequenced using BigDye Terminator kits v.3.1 (Applied Biosystems/Life Technologies, Waltham, MA, USA). The PCR products were electrophoresed using an ABI 3130x1 Genetic Analyzer (Applied Biosystems/Life Technologies), and DNA sequences were compared with GenBank (http://www.ncbi.nlm.nih.gov) reference DNA sequences: NT_0025028.14 and NT_005403.17 for genomic sequences of *ATP8B1* and *ABCB11*, respectively; and NM_005603.4 and NM_003742.2 for *ATP8B1* and *ABCB11* mRNAs, respectively. In silico prediction of the effects of missense variants in *ATP8B1* and *ABCB11* were performed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and Sorting Intolerance from Tolerant (SIFT; http://sift.jcvi.org).

**RESULTS**

**Clinical and laboratory findings**

The clinical characteristics of the five patients with PFIC are listed in Table 1. One was diagnosed with PFIC1 and four with PFIC2. Four were female and one was male. Age at PFIC onset was birth in one patient with PFIC1 and three with PFIC2, and 45 days in a fourth patient with PFIC2. Four patients were Korean and one was Arabian. The Arabian girl had been previously diagnosed with PFIC2 and underwent LT at another hospital. Jaundice was common to all five patients (100%). Only one PFIC2 patient had pruritus in addition to cholestasis. The median total bilirubin and direct bilirubin concentrations at the time of diagnosis were 19.6 mg/dL (range, 10.0 to 24.4 mg/dL; normal range, 0.2 to 1.2 mg/dL) and 15.0 mg/dL (range, 5.6 to 21.0 mg/dL; normal range, < 0.5 mg/dL), respectively. All patients had low or normal GGT (median, 30.0 mg/dL; range, 15.0 to 57.0 mg/dL; normal range, 8 to 61 mg/dL). Four patients, one with PFIC1 and three with PFIC2, underwent LT, followed by allograft liver biopsies performed two or three times.
Histopathological findings

Histopathological features of the five patients with PFIC are shown in Table 2. All had intrahepatic cholestasis, but the patterns of cholestasis were different in patients with PFIC1 and PFIC2. The main finding in the patient with PFIC1 was bland lobular cholestasis with bile plugs (Fig. 1A), whereas the main findings in the patients with PFIC2 were canalicular, periportal, and/or cholangiolar cholestasis (Fig. 1B). Ballooning degeneration of hepatocytes (Fig. 1C) and giant cell transformation (Fig. 1D) were observed in three and three patients with PFIC2, respectively. Small cell changes in hepatocytes were observed only in the PFIC1 patient (Fig. 1A). Lobular activity ranged from none to severe, whereas portal activity was mild in the four patients with PFIC2 (75%). Ductular reactions were observed in the one patient with PFIC1 and in four with PFIC2. The stage of fibrosis varied in patients with PFIC2, with one having cirrhosis, two having periportal fibrosis, and one having no portal fibrosis. The PFIC1 patient had periportal fibrosis. Severe lobular fibrosis was observed in three PFIC2 patients (Fig. 2A), and mild fibrosis in one PFIC1 and one PFIC2 patient (Fig. 2B). Electron microscopy showed coarse and granular bile, called Byler’s bile, in the one PFIC1 patient (Fig. 3A), and amorphous bile in four patients with PFIC2 (Fig. 3B).

BSEP immunostaining

BSEP immunohistochemical staining in patient with one PFIC1 showed normal canalicular expression (Fig. 4A). PFIC2 showed focal loss of BSEP expression in two patients (Fig. 4B) and total loss of BSEP expression in two patients (Fig. 4C), with the latter having an ABCB11 gene mutation.

Genetic mutation analysis

Genetic testing (Table 3) showed a nonsense mutation, c.1753G>T (p.E585*), in ATP8B1 in the one patient with PFIC1. Genetic data were available from all PFIC2 patients. Two patients had mutations in ABCB11, with one having a missense mutation, c.1907A>G (p.E636G), and the other having a nonsense mutation, c.1416T>A (p.Y472*). No mutations on ABCB11 gene were identified in two cases of PFIC2. In addition, the PFIC1 patient had a missense mutation, c.2246T>C (p.L749P) and one PFIC2 patient had a missense mutation, c.2594C>T (p.A865V) in ABCB11.
with PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and SIFT (http://sift.jcvi.org) predicting that these missense mutations would lead to functional derangements of their respective proteins. These novel mutations could not be validated to exclude polymorphisms.

**Clinicopathological course**

An abdominal computed tomography scan in one PFIC2 patient before LT showed multiple enhancing nodules and masses in both lobes of the liver. These were histologically found to be hepatocellular carcinomas and multiple infantile hemangiomas. Recurrence following LT was identified in the one Arabian PFIC2 patient. This patient experienced malnutrition and intractable jaundice 12 years after LT. Laboratory tests showed that, despite markedly elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, and direct bilirubin concentrations, her GGT stayed in the normal range. Liver biopsy showed canicular and periportal bile plugs, as well as diffuse giant cell and ballooning changes in hepatic lobules. Masson trichrome staining showed periportal fibrosis, and BSEP immunostaining showed complete loss of BSEP protein from the canalicular membrane.

The one patient diagnosed with PFIC1 did not show the expected recovery of AST and ALT concentrations after LT. A posttransplant liver biopsy showed severe fatty changes, ballooning degeneration, and mild lobular activity accompanied

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**Fig. 1.** Histologic findings in patients with progressive familial intrahepatic cholestasis (PFIC). (A) Bland canalicular cholestasis and small cell change of hepatocytes with lobular disarray in our PFIC-1 patient. Bile duct proliferation with cholangiolar cholestasis (B), ballooning change (C), and giant cell transformation of hepatocytes (D) in patients with PFIC-2.
Fig. 2. Stages of fibrosis in patients with progressive familial intrahepatic cholestasis (PFIC). (A) Cirrhosis with diffuse lobular fibrosis in a PFIC2 patient, as shown by Masson trichrome staining. (B) Periportal fibrosis with mild lobular fibrosis in the one patient with PFIC1.

Fig. 3. Electron microscopic findings in patients with progressive familial intrahepatic cholestasis (PFIC). (A) Dilated canaliculi (arrows) with coarse granular bile in the PFIC1 patient. (B) Amorphous and dense bile in PFIC2 patients.

by sinusoidal and perivenular fibrosis. The final diagnosis was steatohepatitis, a frequent complication of LT in PFIC1 patients. One PFIC2 patient each developed acute cellular rejection and ischemic liver damage. All patients were alive at the last follow-up.

**DISCUSSION**

Although PFIC is a relatively well-documented disease worldwide, there have been only two case reports on PFIC in Korean patients. Identification of a mutation in an associated gene is not mandatory for diagnosis of PFIC, but is an important diagnostic tool. However, clinical segmentation remains useful because, although PFIC has been reclassified according to molecular criteria, genetic testing is not always readily available. To our knowledge, this is the first study describing overall features of patients in Korea with PFIC.

PFIC is a heterogeneous group of liver diseases, but laboratory findings are similar in PFIC1 and PFIC2 patients, such as low GGT despite direct hyperbilirubinemia. These results suggest that hyperbilirubinemia in PFIC is associated with bile excretion by hepatocytes. By contrast, common forms of neonatal cholestasis are characterized by increased GGT, usually from damage to the
bile ducts caused by the detergent effect of bile salts.\(^{10,11}\) Although PFIC is unfamiliar to general pediatricians and pathologists, it is not a rare disease, accounting for 10%–15% of patients with neonatal cholestasis of unknown etiology. Knowledge of laboratory findings characteristic of PFIC narrows the differential diagnoses, enabling early diagnosis of PFIC in patients with intrahepatic cholestasis.

Some histopathologic features differ in PFIC1 and PFIC2.\(^{12}\) For example, PFIC1 is characterized by a bland lobular cholestasis with hepatocellular and canalicular patterns, as well as small cell changes in hepatocytes in early life biopsies.\(^{13}\) Because our PFIC1 patient showing small cell change was 2 years old at the time of LT, the presence of small cell change is not limited to infancy but appears over time. Giant cell transformation of hepatocytes and ballooning change with cholestasis were major histologic features in PFIC2 patients, although the amount and distribution of giant cells and ballooning changes vary.\(^{14}\)

In contrast to PFIC2, abnormalities of the interlobular bile ducts are not observed in PFIC1.\(^2\) However, ductular reactions may appear in the latter, indicating disease progression.\(^{12}\) The ductular reaction in our one PFIC1 patient may reflect her relatively older age.

Liver fibrosis is an early event of PFIC, with the grade and pattern of fibrosis influenced by patient age and type of PFIC. Lobular fibrosis may develop in PFIC1 and PFIC2, whereas portal fibrosis is a predominant feature of PFIC2.\(^{15}\) Portal fibrosis may be more associated with ductular reaction and portal pathology of PFIC2.

Electron microscopy has shown that bile properties differ in PFIC1 and PFIC2. Coarse granular bile, called Byler's bile, is a distinct characteristic of PFIC1 and was used to diagnose our patient.\(^{16}\) By contrast, PFIC2 is characterized by nonspecific fine bile located within dilated canaliculi, similar to findings in patients with intrahepatic cholestasis of various etiologies. Electron microscopic examination should therefore be included in the differential diagnosis of pediatric patients with cholestatic liver diseases.

About 80 and 100 genetic mutations have been identified in PFIC1 and PFIC2 patients, respectively.\(^{17,18}\) Despite PFICs being autosomal recessive hereditary diseases, compound heterozygous or homozygous mutations may be associated with structural and functional defects. More severe forms of PFIC are likely associated with homozygous frame shift and nonsense mutations, as well as large genomic deletion. By contrast, a milder form of PFIC, benign recurrent intrahepatic cholestasis, is more likely associated with heterozygous missense mutations.\(^{19}\)

Most point mutations in PFIC1 and PFIC2 are missense, nonsense, and splicing mutations. Structurally abnormal proteins in PFIC1 are due to frame shift (26%), splice site (18%), and nonsense (13%) mutations, as well as large genomic dele-

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**Table 3. Results of genetic testing and BSEP immunostaining**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>PFIC type</th>
<th>Gene</th>
<th>Mutation</th>
<th>Mutation type</th>
<th>BSEP immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>ABCB11 on 2q24</td>
<td>Absent</td>
<td>NA</td>
<td>Total loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c.2594C&gt;T (p.Ala865Val) (p.A865V)</td>
<td>Missense, heterozygous</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>ABCB11 on 2q24</td>
<td>Absent</td>
<td>NA</td>
<td>Total loss</td>
</tr>
<tr>
<td>4*</td>
<td>2</td>
<td>ABCB11 on 2q24</td>
<td>c.1416T&gt;A (p.Tyr472*)) (p.Y472*)</td>
<td>Nonsense, homozygous</td>
<td>Focal loss</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c.2594C&gt;T (p.Ala865Val) (p.A865V)</td>
<td>Missense, heterozygous</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>ATP8B1 on 18q21</td>
<td>c.1753G&gt;T (p.Glu585*) (p.E585*)</td>
<td>Nonsense, heterozygous</td>
<td>Intact</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c.2246T&gt;C (p.Leu749Pro) (p.L749P)</td>
<td>Missense, heterozygous</td>
<td></td>
</tr>
</tbody>
</table>

BSEP, bile salt export pump; PFIC, progressive familial intrahepatic cholestasis; NA, not available.
*Possible mutation; *Arabian.
tion (3%). Functionally deficient proteins with normal structure in PFIC1 are caused by missense mutations (38%) and small inframe deletions (3%). The PFIC1 patient in the present study was found to have two heterozygous mutations; one nonsense, c.1753G > T (p.E585*), and one missense, c.2246T > C (p.L749P), mutation. Heterozygous mutations have been associated with good prognosis and low penetrance in PFIC. However, our PFIC1 patient required an LT at age 26 months, suggesting that heterozygous mutations could not guarantee a benign clinical course in PFIC1 patients.

We failed to detect ABCB11 mutations in two PFIC2 patients in the present study. Fewer than 10% of PFIC patients have no or monoallelic mutations. Mutations in these patients may be present in regulatory domains, untranslated regions, and introns, which cannot be tested by present methods. Despite the absence of mutations or the presence of a single heterozygous ABCB11 mutation, the diagnostic sensitivity of clinical and pathologic findings with negative BSEP immunostaining is approximately 90%. Therefore, the absence of ABCB11 mutations cannot exclude PFIC2.

Ursodeoxycholic acid (UDCA) is considered an initial treatment for patients with PFICs. UDCA can resolve symptoms in PFIC1 patients, but PFIC2 patients generally respond poorly, suggesting that UDCA has uncertain effects on the progression of liver disease. In early stages of PFIC, partial biliary diversion that causes an unloading of bile acid may delay LT. LT is the most efficient and last therapeutic option in patients presenting with liver failure. The selection criteria for LT candidates do not differ from those in patients with other liver diseases. Major indications for LT include end stage liver disease, concurrent hepatocellular carcinoma, and intractable pruritus with no response to biliary diversion. Although LT results in remission in 75%—100% of patients, regardless of PFIC type, specific complications and relapse of disease should be carefully considered prior to LT. Patients with PFIC1 may experience exacerbation of extrahepatic manifestations, including chronic diarrhea and liver steatosis. For example, our one PFIC1 patient experienced rapid development of steatohepatitis not observed in the explant liver.

In summary, PFIC is not infrequently encountered in practice. Early detection and management should be based on proper clinicopathologic correlation and mutation analysis. Because it is more cost-effective, BSEP immunostaining should be performed prior to genetic testing to determine the type of PFIC. Mutation analysis of the ABCB11 gene should be performed in patients with portal tract abnormalities and reduction or loss of BSEP expression on immunostaining. In addition, electron microscopy should always be considered for the diagnosis and typing of PFIC.

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

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Primary Necrobiotic Xanthogranulomatous Sialadenitis with Submandibular Gland Localization without Skin Involvement

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Necrobiotic xanthogranuloma (NXG) is a histiocytic disease, commonly found in the subcutaneous tissue of the periorbital area. To the best of our knowledge, only one case of NXG and three cases of primary xanthogranulomatous sialadenitis have been reported. NXG is considered a systemic disease that may involve the skin and extracutaneous sites, including the lung, myocardium, larynx, pharynx, intestines, and even ovaries. Many NXG patients have shown associated paraproteinemia and monoclonal gammopathy, and there are currently no standardized treatment modalities. Here, we report a rare case of localized primary necrobiotic xanthogranulomatous sialadenitis that presented as a fibrotic palpable mass without skin lesion. This is the first case involving the submandibular gland and that was preoperatively regarded as a tumoral mass.

CASE REPORT

Clinical summary

A 73-year-old woman presented with a painless palpable left chin mass, which had persisted for the previous eight months. There was no accompanying skin lesion. She had no significant medical history, except hypertension, and denied any history of trauma to the left side of the chin. Several conglomerated high density lesions that involved periglandular soft tissue and measured 1.7 cm were found in the left submandibular gland using head and neck computed tomography (Fig. 1A). Fine needle aspiration was performed (Fig. 1B). During the 3-month follow-up period, the palpable mass increased from 1.7 to 2.8 cm; therefore, the patient underwent surgery. An ill-defined mass located in the submandibular gland and periglandular soft tissue was noted in the operative field. Partial resection was performed because it was impossible to achieve complete resection due to severe adhesion. She was treated with anti-inflammatory medication; however, the mass kept increasing in size and was measured at 4 cm at 15 months. She underwent a second operation of wide excision of the salivary gland mass and ipsilateral neck node dissection. During the 12-month postoperative period, no grossly remarkable change in size and no abnormalities in blood
chemistry were noted. She is currently on a follow-up plan and will be seen every 6 months.

**Pathological findings**

Fine needle aspiration revealed numerous polymorphous lymphoid cells with scattered epithelioid histiocytes and tingible body macrophages (Fig. 1B). A partially resected specimen was assessed and found to be composed of one reactive follicular hyperplastic lymph node and fragmented soft tissues that showed chronic inflammation with a collection of foamy histiocytes. Occasional granulomas and giant cells were also noted. There was no evidence of malignancy or infection. Later, a fibrotic and very adhesive

![Fig. 1. Necrobiotic xanthogranulomatous sialadenitis. (A) Head and neck computed tomography shows several conglomerated masses in the submandibular gland. (B) Fine needle aspiration cytology reveals many lymphocytes and scattered histiocytes with tingible bodies (PAP). (C) Gross picture of the resected specimen shows a well-circumscribed, ovoid, white-tan mass with yellowish necrotic foci. (D) Necrobiotic xanthogranuloma shows a zone of thick collagen surrounded by lymphohistiocytic palisading granulomas. (E) Foreign body type and Touton-type giant cells are noted (CD68 immunostaining, inset). (F) Cholesterol clefts (upper center) and foam cells are seen adjacent to necrobiosis.](http://jpatholtm.org/)
salivary gland was widely excised. The cut surface showed a relatively well-demarcated, grayish-white, ovoid-shaped mass that measured 3.5 × 2.5 × 1.5 cm (Fig. 1C). Microscopically, the firm lesion was composed of an admixture of foamy macrophages, plasma cells, and many lymphoid follicles. Extensive band-like collagenous geographic necrosis was noted (Fig. 1D). Both Touton- and foreign body type giant cells were abundant in the periphery of the necrosis (Fig. 1E, F). The growth pattern of the mass was infiltrative with adjacent fibroadipose tissue and skeletal muscles, sparing the lymph nodes. Lymphoplasmacytic infiltration was found in the background of the salivary gland parenchyma. The histiocytic cells were positive for CD68 (KP1, 1:100 dilution, DAKO, Glostrup, Denmark) and negative for CD1a (MTB1, 1:100 dilution, Novocastra, Newcastle upon Tyne, UK) (Fig. 1E, inset). No microorganisms were noted on the acid-fast bacilli, periodic-acid Schiff, or Gomori-methenamine silver stains. No increase in the number of IgG4-positive plasma cells (IgG4, 1:1,000 dilution, Invitrogen, Carlsbad, CA, USA) was found at maximum one positive cell/high power field. The aforementioned histologic findings were compatible with the diagnosis of NXG.

Approval was obtained from our Institutional Review Board (No. GBIRB2018-018) for this case report, with a waiver of informed consent.

**DISCUSSION**

NXG is a rare histiocytic disease that involves the skin and subcutaneous tissue. Common sites include the periocular area and thorax. NXG is rare in the head and neck area, and there has been only one case reported in the salivary gland. NXG has characteristic histologic features, showing clusters of foamy xanthomatosus macrophages, granulomas, and giant cells of foreign body type or Touton-type, cholesterol clefts, and necrobiosis. The most characteristic findings are extensive necrosis and inflammation with foamy macrophages and giant cells. These features are similar to the findings of previously reported cases of xanthogranulomatous sialadenitis. Although there was a lack of detailed information about degree of necrosis, infiltration of foamy cells, and amount of giant cells accumulation, we hypothesize that there is an overlap of xanthogranulomatous sialadenitis and salivary NXG. Reports of xanthogranulomatous sialadenitis are rare, and only six cases have been reported to date. When excluding the secondary changes of preexisting salivary gland lesions, including Warthin tumor, pleomorphic adenoma, and sialolithiasis, only three cases of xanthogranulomatous inflammation were
identified in the literature. No cutaneous lesions were noted in this or previous cases. Three cases were misdiagnosed as Warthin tumor in preoperative cytology, and this misdiagnosis can be attributed to the aspiration cytology that included a mixed population of lymphoid cells and presence of histiocytes of oncocytic and epithelioid features. These are summarized in Table 1.

NXG is in a category of other histiocytosis syndromes (category D76.3) that has been outlined by the World Health Organization International Classification of Diseases, 10th revision classification system of diseases. Histologic differential diagnoses of NXG include histiocytic lesions, such as necrobiosis lipoidica diabeticorum, juvenile xanthogranuloma, granuloma annulare, foreign-body granuloma, rheumatoid nodules, amyloidosis, xanthoma disseminatum, and Erdheim-Chester disease. The histiocytes seen in NXG are CD68-positive and CD1a-negative, with no Birbeck granules, which correspond to a non–Langerhans cell histiocytosis. However, a pathologic diagnosis of NXG may be challenging due to its occasionally uncharacteristic features that overlap those of necrobiosis lipoidica diabeticorum. Cholesterol clefts that are more commonly found in NXG can be helpful for ruling out other inflammatory granulomatous lesions. This should be distinguished from necrobiosis lipoidica diabeticorum and Erdheim-Chester disease by characteristic histology and through clinicoradiologic findings.

The pathogenesis of NXG is still unclear; one hypothesis suggests that the serum monoclonal protein functions as a lipoprotein and induces xanthogranulomatous reaction. Another theory suggests that necrosis leads to ischemia. Some reported cases showed an increased number of IgG4-positive plasma cells. One study has indicated the presence of spirochetal microorganisms. There was no evidence of increased IgG4 deposition in the tissue or serum in this case, and no abnormal findings were noted in blood chemistry. We were not able to detect Borrelia by focus-floating microscopy. Some authors have asserted that fine needle aspiration may induce ischemia. Fine needle aspiration of the salivary glands is commonly performed; however, there are extremely rare findings of extensive necrosis with xanthogranulomatous inflammation in surgical specimens.

The clinical course of NXG is variable and unpredictable, and recurrence of the lesion and/or multiple dissemination have been reported at other sites. Other cases have shown systemic involvement, including hematologic malignancy and cardiac myopathy. About 20% of patients in a previous study developed hematologic malignancy during the 11-year follow-up period. Although most of these involved an ocular lesion, one case that involved the salivary gland showed an emergence of lymphoma after multifocal and multiple recurrence of NXG. Therefore, although there is no evidence of hematologic malignancy to date, close follow-up is mandatory.

Due to its rarity, there is currently no standardized treatment guideline for NXG, although treatment approaches have included chemotherapy, interferon, plasmapheresis, steroid injections, cryotherapy, radiation, and surgery. In previous cases of necrobiotic xanthogranulomatous sialadenitis, chemotherapy, radiation, and surgical removal have been attempted. More than one of four patients in a previous study experienced NXG recurrence after surgery. Alkylating agents may be proposed, and steroids can be used to maintain remission. However, it is difficult to achieve complete excision due to adhesion; therefore, additional therapy after surgery is needed in cases without systemic involvement.

Although NXG has distinct pathologic findings, due to its rarity, diagnosing salivary gland NXG remains challenging. Therefore, it is important for clinicians to consider this diagnosis when a patient presents with recurrent adhesive mass with extensive necrotic change. When a diagnosis is made, further evaluation and close follow-up are needed to identify any potentially hidden hematologic malignancies, such as paraproteinaemia and lymphoma, even without manifestation of cutaneous or systemic NXG. Here, we reported a rare case of progressing primary necrobiotic xanthogranulomatous sialadenitis with no accompanying skin lesion or hematologic malignancy.

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Conflicts of Interest
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Rectal Invasion by Prostatic Adenocarcinoma That Was Initially Diagnosed in a Rectal Polyp on Colonoscopy

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Case Study

Prostatic adenocarcinomas spread very slowly, although bone metastasis is common if the cancer is untreated. Prostatic adenocarcinoma with rectal invasion is extremely rare because of the rectoprostatic fascia (Denonvilliers’ fascia) between the prostate and anterior rectal wall.1-3 Because of its rarity, prostatic adenocarcinoma with rectal invasion can be misdiagnosed as rectal adenocarcinoma on colonoscopic biopsy specimens. Here, we report a rare case of a patient with prostatic adenocarcinoma that was initially diagnosed from biopsy of a rectal polyp.

Case Report

A 69-year-old Korean man presented with dull anal pain and voiding discomfort for several days in July 2018. His past medical history included diabetes mellitus treated with standard medications for several years. He showed no signs on the urinary system examination including digital rectal examination, prostate specific antigen (PSA) test and urinalysis a year ago, and he was relatively healthy until recently. Furthermore, he was relatively healthy, until recently. His recent tumor marker test results revealed the PSA level was within the normal limit at 3.25 ng/mL (normal range, 0.04 to 4.0 ng/mL), and his carcinoembryonic antigen (CEA) level was significantly elevated at 18.26 ng/mL (normal range, 0 to 3 ng/mL). A computed tomography scan revealed an abnormally enhancing mass in his entire prostate that extended into the anterior wall of the distal rectum and the urinary bladder, and multiple lymphadenopathy was also observed (Fig. 1A). Because the possibility of rectal adenocarcinoma with prostatic invasion could not be excluded, the patient underwent colonoscopy to distinguish between prostate cancer and rectal cancer. Colonoscopy revealed a protuberant, 1.5-cm-sized, sessile polyp that was located 1.5 cm above the anal verge (Fig. 1B). A microscopic examination of the rectal biopsy specimen revealed a glandular architecture with prominent nucleoli that infiltrated the rectal mucosa and submucosa (Fig. 2). However, no evidence of intraepithelial neoplasia was found in the rectal mucosa. Immunohistochemical staining demonstrated alpha-methylacyl-CoA-racemase (P504s) positivity, PSA weak positivity, and caudal-related homeobox 2 negativity. The final diagnosis of the rectal polyp was consistent with prostatic adenocarcinoma. Here, we present a rare case that could have been misdiagnosed as rectal adenocarcinoma.

Key Words: Prostatic adenocarcinoma; Colonoscopy; Rectum; Polyp

Despite anatomical proximity, prostatic adenocarcinoma with rectal invasion is extremely rare. We present a case of rectal invasion by prostatic adenocarcinoma that was initially diagnosed from a rectal polyp biopsied on colonoscopy in a 69-year-old Korean man. He presented with dull anal pain and voiding discomfort for several days. Computed tomography revealed either prostatic adenocarcinoma with rectal invasion or rectal adenocarcinoma with prostatic invasion. His tumor marker profile showed normal prostate specific antigen (PSA) level and significantly elevated carcinoembryonic antigen level. Colonoscopy was performed, and a specimen was obtained from a round, 1.5 cm, sessile polyp that was 1.5 cm above the anal verge. Microscopically, glandular tumor structures infiltrated into the rectal mucosa and submucosa. Immunohistochemically, the tumor cells showed alpha-methylacyl-CoA-racemase positivity, PSA positivity, and caudal-related homeobox 2 negativity. The final diagnosis of the rectal polyp was consistent with prostatic adenocarcinoma. Here, we present a rare case that could have been misdiagnosed as rectal adenocarcinoma.
related homeobox 2 (CDX-2) negativity (Fig. 3). The tumor cells were consistent with a prostatic origin; thus, the final diagnosis of the rectal polyp was prostatic adenocarcinoma (Gleason score 4 + 4). After diagnosis of prostatic adenocarcinoma, the patient refused all procedures and treatment and was transferred to another hospital. Ethical approval was exempted for this case report by the Institutional Review Board of Kyungpook National University Medical Center, and the need for informed consent was waived through the de-identification of all the patient’s personal information (No. KNUCH 2019-01-034).

DISCUSSION

Prostate cancer is a major contributor to cancer-related mortality and morbidity, despite its slow progression. Prostate cancer commonly metastasizes to the bones and lymph nodes, but metastases to the digestive tract are relatively rare. In addition, prostate cancer frequently directly infiltrates into the bladder and ureters but rarely invades the rectum due to Denovilliers’ fascia, a membranous barrier at the lowest part of the rectovesical pouch that separates the prostate and urinary bladder from the rectum and inhibits posterior spread of prostatic adenocarcinoma. Three potential routes have been proposed for invasion of prostate cancer into the rectal wall: (1) direct invasion through Denovilliers’ fascia; (2) lymphatic metastasis into the rectum; and (3) iatrogenic spread of cancer cells that seed into peri-rectal or rectal tissue along a needle biopsy tract. Our patient had no history of needle biopsy of the prostate. Unfortunately, prostate cancer with rectal invasion is occasionally confused with a primary rectal cancer because of their similar radiologic appearance, clinical presentation, and morphologic features. Notably, it might be difficult to differentiate prostatic adenocarcinoma from rectal adenocarcinoma in small rectal biopsy specimens. Tang et al. reported 9,504 patients, nine of whom had prostatic adenocarcinoma involving the rectal wall and were clinically and pathologically misdiagnosed with rectal adenocarcinoma.

Fig. 1. (A) Abdominopelvic computed tomography scan reveals an abnormally enhancing mass in the prostate gland that invaded the urinary bladder, both seminal vesicles, and the anterior wall of the distal rectum. (B) Representative image of the colonoscopy shows a sessile rectal mass about 1.5 cm from the anal verge.

Fig. 2. (A) Representative image of hematoxylin and eosin staining of the rectal biopsy specimen. (B) Tumor cells had prominent nucleoli.

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carcinoma. This risk of misdiagnosis could result in inappropriate treatment strategies and lead to adverse consequences for the patient. Although PSA level might help distinguish these two entities, PSA is not expressed in all patients with prostatic adenocarcinoma.\(^7,9\) In our case, the PSA level was within normal limits, whereas the CEA level was significantly elevated, and the possibility of rectal adenocarcinoma could not be excluded.

Because several cases of extracolonic cancer have presented as colorectal polyps, consideration of a wide range of differential diagnoses is warranted.\(^10\) Delicate histologic differences between prostate and rectal cancers could facilitate accurate diagnoses. In the present case, the tumor consisted of small- or medium-sized glands with cribriform proliferation, and the cancer cells showed mild nuclear enlargement with prominent nucleoli. Unlike prostatic adenocarcinoma, the histologic features that favor rectal adenocarcinoma include tall columnar cells with basally located nuclei, ‘dirty’ necrosis, villous architecture, and stromal reaction. Furthermore, the immunohistochemistry of specific markers for these two entities helps confirm tumor origin. PSA and P504s are highly sensitive and specific commonly-used positive markers for prostatic adenocarcinoma.\(^2,11,12\) However, PSA and P504s are not always expressed in prostatic adenocarcinoma, and they show variable expression levels depending on tumor differentiation.\(^13\) Moreover, our patient showed weak PSA positivity but strong P504s positivity. In contrast, rectal adenocarcinoma commonly shows CDX-2 and CEA positivity but PSA and P504s negativity. Notably, some cases with androgen-independent prostatic adenocarcinoma displaying CDX-2 positivity have been reported,\(^14\) demonstrating one of the potentially dangerous pitfalls for differentiating rectal adenocarcinoma.\(^2\) Conventionally, prostatic adenocarcinoma with rectal invasion represents an advanced stage and has a poor prognosis.\(^5\) In particular, rectal invasion is frequently a sign of more widespread systemic disease, which highlights the importance of a timely and accurate diagnosis. Above all, misdiagnosis as rectal adenocarcinoma can lead to inappropriate resection, such as abdominoperineal excision and anterior resection of the rectum.

In summary, we described a case of prostatic adenocarcinoma that presented with rectal symptoms and was initially diagnosed in a rectal polyp on colonoscopy. The patient had no previous medical history of prostatic adenocarcinoma, and his PSA level was within normal limits. Based on our experience, this rare finding might help clinicians and pathologists avoid misdiagnoses when faced with such cases in colorectal practice.

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**Conflicts of Interest**

The authors declare that they have no potential conflicts of interest.
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Endocervical Adenocarcinoma In Situ Phenotype with Ovarian Metastasis

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Adenocarcinoma in situ (AIS) of the endocervix is a precursor of usual type endocervical adenocarcinoma. By definition, endocervical AIS normally involves simple columnar epithelium only and does not invade the cervical stroma. Endocervical AIS exhibits a variable anatomic distribution, extending along the endocervical canal for several centimeters, and sometimes involving the entire circumference of the cervix. Extension of endocervical AIS proximal to the internal orifice is uncommon and metastatic involvement of unilateral or bilateral ovaries by endocervical adenocarcinoma is even rarer. However, several studies have reported a subset of endocervical tumors with minimal to no evidence of stromal invasion manifesting as metastatic ovarian tumors. Distinguishing this metastatic disease from a primary ovarian tumor is difficult because ovarian lesions are typically large, unilateral, confined to the ovary, and well differentiated with mucinous or endometrioid-like histology, which are features consistent with a primary ovarian tumor. The metastatic nature of these lesions can be confirmed by comparing the human papillomavirus (HPV) type to that of the endocervical tumors. In this report, we demonstrate a case of endocervical AIS with no evidence of stromal invasion involving the unilateral ovary and simulating a primary ovarian tumor.

CASE REPORT

A 45-year-old woman presented with a left ovarian mass 65 months following diagnosis of endocervical AIS (Fig. 1A) from a punch biopsy and treated with an extended abdominal hysterectomy. She underwent left salpingo-oophorectomy and the left ovarian mass measured 7.0 cm in diameter, had a smooth surface, and was solid and cystic. The inner surface of the cystic lesion varied from smooth to rough with several nodules. Histologically, the tumor displayed confluent glandular architectures with primarily endometrioid-like (Fig. 1B) and focally mucinous (Fig. 1C) differentiation. Nuclei were enlarged and hyperchromatic with occasional mitotic figures and apoptotic bodies (Fig. 1D). There was no stromal invasion. The ovarian tumor was thought to represent an independent borderline endometrioid tumor. We reviewed slides obtained from the hysterectomy specimen, and concerns were raised for metastasis due to the histological similarity between the endocervical and ovarian lesions. Immunostaining for p16 and HPV genotyping were performed. Both endocervical (Fig. 1E) and ovarian (Fig. 1F) lesions exhibited strong and diffuse p16 expression and had identical HPV type 45. Based on these findings, the left ovarian tumor was diagnosed as metastatic endocervical adenocarcinoma. This study was approved by the Institutional Review Board of Severance Hospital with a waiver of informed consent (4-2018-0724).

DISCUSSION

When histologically similar malignant tumors occur at two locations, it is difficult to determine whether each is an independent primary tumor, or whether one represents a metastasis from the other. The concurrence of endocervical adenocarcinoma and ovarian tumor is quite uncommon, and the diagnostic difficulties encountered have received little attention. The majority of cases with ovarian involvement occur in advanced-stage endo-
cervical adenocarcinoma or in a primary lesion with extraterine extension. However, there have been cases of early-stage disease, and even cases of noninvasive carcinoma, that involved the ovary. Most ovarian lesions show well-differentiated villoglandular and confluent glandular patterns, resembling those of primary ovarian borderline mucinous or endometrioid tumor. In a previous study, all cases examined were initially thought to represent independent primary ovarian epithelial tumors, including borderline tumors or grade 1 carcinomas of mucinous or endometrioid type. In cases with no stromal invasion in an endocervical adenocarcinoma, a borderline-appearing ovarian tumor could reasonably be interpreted as an independent primary ovarian tumor by the pathologist. In this situation, the presence of the identical HPV type in both the endocervical and ovarian tumors supports that the ovarian lesion is a metastatic endocervical adenocarcinoma rather than an independent primary ovarian tumor. Studies associating HPV and ovarian tumors have yielded almost universally negative results, suggesting that primary ovarian tumors are etiologically unrelated to HPV infection. In addition, p16 immunostaining may prove useful for distinguishing metastases from primary ovarian tumors. p16 immunoreactivity is a surrogate marker of high-risk HPV infection. Although p16 expression is absent or sporadically positive in ovarian mucinous and endometrioid carcinomas, HPV-related endocervical tumors typically display strong and diffuse nuclear p16 immunoreactivity. In conjunction with clinicopathological features, p16 expression in ovarian lesions is associated with high-risk HPV and supports the diagnosis of metastatic endocervical adenocarcinoma.

In summary, we present a case of endocervical AIS involving the ovary. Notably, minimal to no stromal invasion in endocervical adenocarcinoma does not exclude the possibility of metastasis to the ovary. Cases of concurrent endocervical AIS and ovarian tumor, although rare, prompt thorough sampling of the endocervix to exclude stromal invasion. Careful morphological evaluation and use of ancillary tests promote an accurate diagnosis. p16 immunostaining and HPV testing of ovarian lesions may be confirmatory if positive.

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**Fig. 1.** (A) Histological findings of the endocervical lesion. Endocervical adenocarcinoma in situ (AIS) extensively involves the endocervix. No cervical stromal invasion is identified. (B–D) Histological findings of the ovarian lesion. The tumor displays a well-differentiated glandular architecture with primarily (B) endometrioid-like and focally (C) mucinous differentiation. (D) High-power magnification of the ovarian lesion reveals enlarged and hyperchromatic nuclei with intracytoplasmic mucin vacuoles, mitotic figures, and apoptotic bodies, similar to those of the endocervical AIS. (E, F) p16 immunostaining results. Both the (E) endocervical and (F) ovarian lesions show strong and diffuse p16 immunoreactivities.
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Conflicts of Interest

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