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Focal segmental glomerulosclerosis (FSGS) is characterized by focal and segmental obliteration of glomerular capillary tufts with increased matrix. FSGS is classified as collapsing, tip, cellular, perihilar, and not otherwise specified variants according to the location and character of the sclerotic lesion. Primary or idiopathic FSGS is considered to be related to podocyte injury, and the pathogenesis of podocyte injury has been actively investigated. Several circulating factors affecting podocyte permeability barrier have been proposed, but not proven to cause FSGS. FSGS may also be caused by genetic alterations. These genes are mainly those regulating slit diaphragm structure, actin cytoskeleton of podocytes, and foot process structure. The mode of inheritance and age of onset are different according to the gene involved. Recently, the role of parietal epithelial cells (PECs) has been highlighted. Podoceytes and PECs have common mesenchymal progenitors, therefore, PECs could be a source of podocyte repopulation after podocyte injury. Activated PECs migrate along adhesion to the glomerular tuft and may also contribute to the progression of sclerosis. Markers of activated PECs, including CD44, could be used to distinguish FSGS from minimal change disease. The pathogenesis of FSGS is very complex; however, understanding basic mechanisms of podocyte injury is important not only for basic research, but also for daily diagnostic pathology practice.

Key Words: Focal segmental glomerulosclerosis; Podocytopathy; Permeability factors; Parietal epithelial cells
There are several observations indicating that podocyte injury is at the center of the development of FSGS. First, podocyte injury is the earliest morphologic feature of FSGS. In recurrent FSGS in the allograft kidney, podocyte injury is detected by electron microscopy prior to the development of overt sclerosis. Second, there are animal models of podocyte-specific injury resulting in FSGS. NEP25 mice express human CD25 specifically on podocytes. Injection of immunotoxin which binds to human CD25 induced podocyte-specific injury and FSGS occurred a few weeks later. Rats expressing diphtheria toxin receptors on podocytes developed FSGS after diphtheria toxin injection. Third, histologic appearance of FSGS and clinical symptoms were in proportion with the number of injured podocytes. Therefore, the pathogenesis of podocyte injury is a key to understand the characteristics of FSGS.

**CIRCULATING PERMEABILITY FACTORS**

As for the pathophysiology of podocyte injury, several mechanisms have been proposed with supporting evidences. Circulating permeability factors have been reckoned as the initiating factor of podocyte injury in primary FSGS and its recurrence after transplantation. The presence of serum factors that can cause podocyte injury was suggested from the therapeutic effect of immunoadsorption therapy and observations that plasmapheresis could decrease the glomerular injury induced by patients' serum. Further, serum of recurrent FSGS patients significantly increased albumin permeability of glomeruli in an *in vitro* test. Among the proposed circulating permeability factors, soluble urokinase receptor (suPAR) has been most thoroughly investigated. Wei et al. presented data in a mouse model suggesting that the urokinase receptor of podocytes contributed to podocyte loss and proteinuria. The same group also suggested that suPAR could be the cause of FSGS. Serum levels of suPAR were increased in about two-thirds of primary FSGS patients and were also associated with recurrent FSGS after transplantation. They also demonstrated that suPAR was increased in two different cohorts of biopsy-proven FSGS patients. However, suPAR was inversely correlated with estimated glomerular filtration rate (eGFR) and treatment response. Other authors found that plasma suPAR level was significantly increased in FSGS patients versus patients with minimal change disease, membranous nephropathy, or normal control. However, suPAR level was not useful in distinguishing primary and secondary FSGS. Several contradicting reports on suPAR have also been published. Importantly, eGFR affects plasma levels of suPAR in patients with non-FSGS glomerular lesions, and a suPAR cut off value could not be determined even in FSGS patients due to the effect of eGFR. Plasma levels of suPAR were also increased in lupus nephritis patients compared to lupus patients without renal involvement. In IgA nephropathy patients, the plasma level of suPAR was related to the development of secondary segmental sclerosis. Therefore, whether suPAR plays a role in the development of focal segmental lesions and its specificity to the primary FSGS are still open to further investigation.

Cardiotrophin-like cytokine-1 (CLC-1 or cardiotrophin-like cytokine factor 1 [CLCF-1]) is another candidate circulating permeability factor for primary FSGS. Savin et al. have published on a serum factor purified from FSGS patients, which increased albumin permeability in isolated rat glomeruli. This factor had affinity for galactose and its molecular weight was less than 30 kDa. They identified this factor as CLC-1 by proteomic analysis and also found that the activity of CLC-1 was decreased by several factors such as heterodimer formation with cosecreted cytokine receptor-like factor 1 (CRLF1), Janus kinase 2 (JAK2) inhibitor, and signal transducer and activator of transcription 3 (STAT3).
A phase II clinical trial on therapeutic effect of galactose in patients with steroid-resistant FSGS was performed with inconclusive results due to small sample size. 33,34 This study design is interesting, considering that CLC-1 has high affinity for galactose and the CLC-1–galactose complex can be easily removed in the liver. 30

Though known to be related to minimal change disease rather than FSGS, angiopoietin-like-4 (Angptl4) is also of interest. 35 Angptl4 has different functions according to its sialylation. While proteinuria was induced by hypoosialylated Angptl4 located within the glomerulus, normosialylated Angptl4 was present in the peripheral circulation and mediated hypertriglyceridemia, 35,36 indicating that these two symptoms of nephrotic syndrome could be linked through a common circulating factor.

**GENETIC BACKGROUND**

FSGS, as a podocytopathy, may be caused by mutation in several genes, which are important in maintaining podocyte morphology and function. Most of these genes can be categorized as those which are related with slit diaphragm structure, actin cytoskeleton of podocytes, or podocyte-glomerular basement membrane interaction through foot processes. 37,38 In addition, a specific channel mutation (see below) has also been identified as a cause of FSGS (Table 1). Alteration of these genes results in autosomal dominant or recessive congenital, infantile, or late onset nephrotic syndrome, some of which presents as FSGS histologically. 40 Mutation in the NPHS1 gene and resulting loss of its product nephrin, are responsible for congenital nephrotic syndrome of Finnish type. The locus of NPHS1 was identified at 19q13.1 in 1998, 41 which was the first identification of a podocytopathy-related gene. After this discovery, NPHS2, 42,43 PLCE1 (phospholipase Cε1, NPHS3), 43 WT1 (Wilms tumor 1, NPHS4), 44 LAMB2 (laminin β2, NPHS5), 45 PTPRO (protein tyrosine phosphatase receptor type O, NPHS6), 46 ARHGDI A (Rho GDP dissociation inhibitor α, NPHS8), 47 ADCK4 (aaf domain containing kinase 4, NPHS9), 48 and EMP2 (epithelial membrane protein 2, NPHS10) 49 were identified and related to autosomal recessive nephrotic syndrome. Many other genes related to nephrotic syndrome have been identified including ACTN4 (actinin α4, FSGS1), 50 TRPC6 (transient receptor potential cation channel 6, FSGS2), 51 CD2AP (CD2-associated protein, FSGS3), 52 APOL1 (apolipoprotein L1, FSGS4), 53 INF2 (inverted formin, FSGS5), 54 MYO1E (myosin 1E, FSGS6), 55 PAX2 (paired box gene 2, FSGS7), 56 ANLN (anillin, FSGS8), 57 and CRB2 (Crumbs homolog 2, FSGS9). 58 There are interactions of these genes and their products. For example, WT1 transcriptionally regulates nephrin encoding of NPHS1, therefore, WT1 mutations influence NPHS1 function. 59 A study in a European cohort reported that two thirds of nephrotic syndrome within 1 year of life are related to alteration of NPHS1, NPHS2, WT1, or LAMB2. 60 Another study in a non-Finnish ethnic group also reported that NPHS1 and NPHS2 mutations were the most common genetic alterations in congenital nephrotic syndrome. 61 In contrast to these Western studies, a genetic analysis of 30 Korean congenital and infantile nephrotic syndrome patients revealed that WT1 and NPHS1 mutations were the most frequent alterations, while NPHS2 mutations were the lowest frequency genetic alteration. 62

**PARIETAL EPITHELIAL CELLS AND PODOCYTE INJURY**

Podocytes are terminally differentiated cells having very limited ability of regeneration or proliferation. Therefore, the mechanism of repopulation of podocytes after podocyte injury has been of great interest. Recently, it has been suggested that parietal epithelial cells (PECs) lining Bowman’s capsule play an important role in this process by migrating from their original site to replace injured podocytes. 63 During glomerulogenesis, PECs and podocytes originate from common mesenchymal progenitors and finally have different phenotypes. Although little is known about the function of terminally differentiated PECs, they express tight junction molecules such as claudin-1, zonula occludens-1, and

| Table 1. Genes related to FSGS or nephrotic syndrome |
|-----------------------------------------------------|
| NPHS1 | Nephrin |
| NPHS2 | Podocin |
| PLCE1 (NPHS3) | Phospholipase Cε1 |
| WT1 (NPHS4) | Wilms tumor 1 |
| LAMB2 (NPHS5) | Laminin β2 |
| PTPRO (NPHS6) | Protein tyrosine phosphatase receptor type O |
| ARHGDI A (NPHS8) | Rho GDP dissociation inhibitor α |
| ADCK4 (NPHS9) | aaf domain containing kinase 4 |
| EMP2 (NPHS10) | Epithelial membrane protein 2 |
| ACTN4 (FSGS1) | α-Actin-4 |
| TRPC6 (FSGS2) | Transient receptor potential cation channel 6 |
| CD2AP (FSGS3) | CD2-associated protein |
| APOL1 (FSGS4) | Apolipoprotein L1 |
| INF2 (FSGS5) | Inverted formin |
| MYO1E (FSGS6) | Myosin 1E |
| PAX2 (FSGS7) | Paired box gene 2 |
| ANLN (FSGS8) | Anillin |
| CRB2 (FSGS9) | Crumbs homolog 2 |

FSGS, focal segmental glomerulosclerosis.
occludin and have barrier function against protein. Some PECs express both CD133 and CD24, which are known to be stem cell markers, and these cells have regenerative ability. More detailed study revealed that PECs show hierarchical differentiation according to their locations. PEGs located at the urinary pole express CD133 and CD24 without the expression of podocyte markers (nestin, complement receptor-1, and podocalyxin). PEGs of the vascular pole express podocyte markers without the expression of CD133 or CD24. In other areas, PEGs express both CD133/CD24 and podocyte markers. CD133 and CD24-expressing PECs have the ability to ameliorate kidney injury by potentiating tubular regeneration and podocyte replacement, however, they can also contribute to glomerular injury such as glomerulosclerosis and crescent formation. Animal models and human post-transplant biopsies demonstrated that invasion of activated PEGs through the adhesion sites of the capillary tuft contributed to the development of FSGS. The adhesion of the glomerular tuft to the Bowman’s capsule as a bridge of PEG migration appears to occur at early stages of FSGS development. Therefore, detecting activated PEGs on Bowman’s capsule or on the glomerular tuft could be an adjunctive diagnostic tool for early FSGS. In support of this concept, CD44 as a marker of activated PECs successfully distinguished early primary FSGS from minimal change disease. Interestingly, mutation of ARHGDIA, which is responsible for nephrotic syndrome, increased migration activity of cultured podocytes.

CONCLUSION

The etiology and pathogenesis of FSGS are very complex. Current research is focusing on the role of podocytes and interaction with PEGs. Understanding the mechanism of podocyte injury, its progression and possible recovery is important not only for basic research but also for daily diagnostic pathology practice.

Conflicts of Interest

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

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Immunohistochemistry (IHC) is an important auxiliary method for pathologists as it specifically visualizes distribution and amount of a certain molecule in the tissue using specific antigen-antibody reaction. The applications of IHC have recently been expanded explosively as more and more molecules involved in pathogenesis, diagnosis, and treatment of diseases are discovered. The unique feature that makes IHC stand out among many other laboratory tests is that it is performed without destruction of histologic architecture, and thus the assessment of an expression pattern of the molecule is possible in the context of microenvironment. The co-analysis of both the target molecule and its subcellular, cellular, and intercellular relation is probably done best by pathologists, and the importance of this co-analysis is increasingly recognized in biomedical research field such as new drug development and prognostic/predictive biomarker investigation. Therefore, pathologists must know thoroughly about the principle and practice of IHC. Here, we aim to provide basic information on procedures and interpretation of IHC with pitfalls and tips for general pathologists.

PROCEDURES OF IMMUNOHISTOCHEMISTRY

There are many critical steps in performing IHC. These include proper handling of the specimen, appropriate fixation, paraffin block preparation, antigen retrieval, selection and preparation of antibody and reagents, incubation, washing, and counterstaining. The advent of automated IHC machines has improved reliability and reproducibility of IHC, particularly in clinical setting. On the other hand, manual staining method still offers more flexibility, allowing for optimization of a specific antigen-antibody reaction, and hence better results, particularly in research setting. Both methods have pros and cons, but basic principles and procedures remain the same. Overall procedure of IHC is summarized in Table 1. Basic principles of each step will follow with practical pitfalls and tips.

Tissue handling and fixation

Ischemia of the resected specimen before fixation results in degradation of protein, RNA, and DNA as well as activation of tissue enzymes and autolysis. Therefore, variation in ischemic time can be a crucial factor affecting IHC results. Alteration in the results of estrogen receptor, progesterone receptor, human epidermal growth factor 2, and Ki-67 IHC due to variable ischemic times has been reported. Fixation is another important cause of variation in the reproducibility of IHC. Surgical specimens are fixed in 10% neutral buffered formalin (NBF). This
process prevents autolysis and preserves tissue and cellular morphology. For most tissues, fixation for 24 hours in room temperature is recommended. The appropriate tissue to fixative ratio is 1:1 to 1:20. Duration of fixation, fixative formula, and tissue to fixative ratio can affect the extent and intensity of IHC.8,9 Fixation is also required in frozen sections in certain situations such as evaluating new antibodies. In those cases, acetone- or NBF-fixed frozen sections can be used.

**Pitfalls and tips**

Some antigens including Ki-67 and phosphoproteins are more vulnerable to ischemia. Overfixation can cause irreversible damage to some epitopes. To avoid ischemic or cold effect resulting in degeneration of protein or tissue enzymes, rapid fixation is important. When using non-additive fixatives such as acetone, the target antigen will normally be fully available, but with compromised morphology. Soluble antigen may be diffused out during the process of IHC if the frozen section is not fixed. IHC should be done with freshly cut sections.

**Antigen (or epitope) retrieval**

Antibody binding epitopes can be masked in formaldehyde-based fixation due to cross-linkings of amino groups on adjacent molecules, in addition to the formation of methylene bridges.13 For this reason, antigen retrieval, an additional step to unmask the epitope, is sometimes required. Optimized antigen retrieval can restore antigenicity to that of frozen sections; thus, antigen retrieval is crucial for IHC standardization for which issues of variations in fixation and handling of specimens must be overcome.14 In general, antigen retrieval process is not necessary for frozen sections. However, sometimes acetone- and NBF-fixed frozen sections are required to prevent wash out of target antigen, particularly for soluble antigens. In that case, antigen retrieval may serve for better IHC signals.

The heat induced epitope retrieval (HIER) is the most widely used method for antigen retrieval. Various methods can be used for application of heat: microwave ovens, heating plate, pressure

**Table 1. Basic protocols of immunohistochemistry**

| Step                          | Protocol                                                                 |
|-------------------------------|--------------------------------------------------------------------------|
| Fixation                      | 10% Neutral buffered formalin for 24 hr in room temperature              |
|                               | Frozen section: cold acetone for 1 min                                   |
| Embedding and sectioning      | Paraffin embedding                                                       |
|                               | Mostly 4 μm                                                              |
|                               | Frozen sections: between 4 μm and 6 μm in thickness                      |
| Deparaffinization and hydration | 60°C hot plate                                                          |
| Antigen (or epitope) retrieval | Heat induced epitope retrieval is most widely used                       |
| Blocking                      | Normal sera of same species of secondary antibody or premixed            |
|                               | Vary from 30 min to overnight, from 4°C to room temperature              |
| Add primary antibody          | Antibody dilution by protein blocking solution or premixed Ab diluents   |
|                               | Appropriate antibody selection and titration                             |
| Incubate                      | 30–60 min, room temperature                                             |
| Wash (TBS-T)                  | 3 × 5 min                                                               |
| Add secondary antibody        | -                                                                       |
| Incubate                      | 30–60 min, room temperature                                             |
| Wash (TBS-T)                  | 3 × 5 min                                                               |
| Add substrate                 | 250 μL of 1% DAB, and 250 μL of 0.3% hydrogen peroxide to 5 mL of PBS, 1–3 minutes, room temperature |
| Wash                          | 3 × 5 min, DW                                                           |
| Counterstain                  | Hematoxylin, 1 min                                                      |

TBS-T, Tris-buffered saline and Tween 20; DAB, diaminobenzidine; PBS, phosphate buffered saline; DW, dextrose 5% in distilled water.

Oxidation by vacuum storage or paraffin coating are important as well as complete removal of water in the slide.

**Pitfalls and tips**

Thick tissue sections can produce higher background signals as can frozen sections; frozen sections tend to preserve more adhesive molecules, Fc receptors, peroxidase, etc. Soluble antigen may be diffused out during the process of IHC if the frozen section is not fixed. IHC should be done with freshly cut sections.

**Tissue sections**

The recommended thickness of tissue section for IHC is mostly 4 μm, but it is optional depending on the purpose. Storage of tissue sections may have influence on the results of IHC;10 storing tissue sections for more than 2 months results in loss of p53.11 The mechanism of this epitope degradation is unclear, but water component in and around the tissue sections may cause the antigenic loss.12 Slide storage conditions that are protected from oxidization by vacuum storage or paraffin coating are important as well as complete removal of water in the slide.
cookers, autoclaves, and water baths in varying conditions including pH 6–10. Generally, using autoclave and microwave oven, the temperature is set at 120°C at full pressure and 750–800 W, respectively and typically for 10 minutes. Using heating plate, incubate at 100°C for 30 minutes. The best retrieval condition for each Ag-Ab pair needs to be determined empirically through comparison of staining results by various retrieval methods. In addition, enzymatic retrieval is used for limited antigens such as some cytokeratins and immunoglobulins. In such cases, tissue sections are incubated in either trypsin or proteinase for 10–20 minutes at 37°C. Then, terminate the reaction by adding phosphate buffered saline (PBS). However, this method is much more difficult to control.

**Pitfalls and tips**

Excessive tissue microwaving can destroy antigenicity and morphology. It may result in HIER lipofuscin artifacts. When using HIER, “microwave burn” pattern in loose connective tissue and fat can be identified. The appropriate antigen retrieval is different from antigen to antigen, and antibody to antibody. It should be determined individually for each antigen and antibody. Also, try enzymatic retrieval or no retrieval in addition to HIER at the initial step.

**Protein blocking**

Protein blocking step is required to reduce unwanted background staining. A main cause for the background signal is the nonspecific binding of Fc portion of primary or secondary antibodies. An ideal agent for the protein blocking is 5%–10% normal serum from the same species of secondary antibody. Other agents include protein buffers such as 0.1%–0.5% bovine serum albumin, gelatin, or nonfat dry milk. Recently commercial mixes of synthetic peptides are also being widely used. Incubation time for the blocking step can vary from 30 minutes to overnight. Incubation temperatures also vary from 4°C to room temperature.

**Pitfalls and tips**

Sufficient washing after the blocking step is critical to remove excess protein that may prevent detection of the target antigen. Choose blocking buffer that yields the highest signal to noise ratio. Nonfat dry milk contains biotin and is inappropriate for use with an avidin-biotin complex system. When using frozen sections, smears and lightly fixed tissues, background staining due to Fc receptor is more prevalent than formalin-fixed, paraffin-embedded sections. Furthermore, it is important to control Fc receptor rich specimens such as lymphoid sections, tonsil sections, and bone marrow preparations. It can be avoided by using either Fc receptor blocking or F(ab')2 fragments of primary staining antibody instead of whole IgG molecules.

**Endogenous enzyme blocking**

When using peroxidase antiperoxidase system in detection step, blocking of endogenous peroxidase activity is indispensable. Diluted hydrogen peroxide as 3% is widely used for blocking endogenous peroxidase activity. Upon completion of the IHC stain, eosinophils and erythrocytes are used as an index to see whether the blocking step has been adequate or not. Similarly, endogenous alkaline phosphatase (AP), which is prevalent in frozen tissue, should be blocked with levamisole at a concentration of 10 mM. The endogenous biotin in tissues is another issue. Although the level of endogenous biotin has been shown to be much lower in formalin fixation and paraffin embedding, residual activity can still be detected, especially in biotin-rich tissues such as liver and kidney. Endogenous biotin can be blocked by incubating tissue section in avidin solution beforehand (incubate sections in avidin solution for 15 minutes followed by brief rinse in PBS, and then incubate sections in biotin solution for 15 minutes, all at room temperature).

**Pitfalls and tips**

Tissues with high blood content (e.g., site of heavy hemorrhage), or with intense granulocytic inflammatory infiltrate, need a strong suppression of endogenous peroxidase activity. Some antigen (such as CD4) can be destroyed by 3% H2O2. In this case, an H2O2 concentration as low as 0.5% is recommended.

**Antibody selection and validation**

Before performing IHC, a selection of suitable antibody is critical, for which understanding of the target through thorough literature review is the first step. If there are reports of the target molecules using IHC, then the antibody used in the previous reports should be evaluated first. Antibodies used in researches are generally divided into three types with respect to the validity and reliability: well-known antibody with high quality literature evidence, well-known antibody used in alternative species or unverified tissues, and unknown antibody with inconsistent or no literature evidence. Researchers should perform an adequate level of validation depending on the kind of antibody used. Detailed guidance for validating antibody is well summarized in the review article.

Antibodies are generally divided into two categories. Polyclonal antibodies are obtained from experimental animals.
through repetitive stimulation of antigen. These antibodies bind to multiple different epitopes in a single antigen. On the other hand, monoclonal antibodies react to a single epitope in an antigen. They are obtained from a single clone of hybridoma, which produces antibodies. Both polyclonal and monoclonal antibodies have advantages and disadvantages, respectively (Table 2). Recently, monoclonal antibodies from rabbit or chicken have been introduced. These antibodies may give a better IHC results for antigens that are difficult to stain.

When an unknown antibody is validated, it is important to select proper positive and negative control tissues. Interpretation of IHC stain pattern in control tissues should be done carefully. Appropriate location, intensity, and signal/noise ratio are determined in this step. Validation of the antibody by non-IHC methods such as western blotting or flow cytometry is also recommended. Afterward, optimization is necessary to tune antibody dilution, incubation times, and blocking for controlled laboratory conditions. Appropriate validation and optimization of IHC staining method can provide equivalent results between laboratories.

**Pitfalls and tips**

The biologic characteristics and amount of the antigen in the tissue, titration of the antibody, and differences between control and testing samples should be carefully considered. Use the same tissue that was used for optimization or validation for testing IHC. Perform IHC with negative control Ab to disclose any background staining. Competition assay with the immunizing peptide on the optimizing sample is essential if the validation level of the antibody is low (see competition protocol in the study of Prioleau and Schnitt).20

**Detection system**

Immunostaining is the process of detecting specific antigen-antibody interaction, and indirect method using secondary antibodies tagged with various labels such as enzymes is commonly used.5 Commonly used detection systems are as follows: avidin-biotin complex method, labeled streptavidin biotin method, phosphatase anti-phosphatase method, polymer-based detection system (Fig. 1), and tyramine amplification system. In comparison to the standard IHC methods, polymeric and tyramine-based amplification methods have typically increased sensitivity by at least 50-fold or greater. The outcome of an IHC depends on the selection of the optimal methods for signal amplification for the molecule of interest and surrounding tissues.21

Another important issue is the use of manual IHC versus automated IHC machines. Automated IHC machine has greatly improved reproducibility and reliability of IHC. However, the automated system does not allow subtle optimization or flexibility in the usage of reagent or retrieval methods. There are advantages and disadvantages.

**Pitfalls and tips**

When more sensitive methods are used, background signal tends to increase along with the target signal. Use automated IHC machine if a large number of samples are tested, or if the samples are tested over a longer time. AP-based detection system is preferred for tissues rich in endogenous peroxidase, such as bone marrow or lymphoid tissue. Likewise, peroxidase based-detection system may be used for tissues containing many endogenous APs but the enzyme can be easily destroyed by high-temperature antigen retrieval. Biotin-free synthetic polymer system is recommended for tissues with high endogenous biotin such as liver and kidney.

Several different chromogens are available according to the type of tissue or counterstain. In general, diaminobenzidine (brown) or 3-amino-9-ethyl carbazole-red are routinely used for peroxidase. The choice depends on the type of tissue or counterstain.

**Counter staining**

Counter staining provides contrast to the chromogens for better discrimination of the target signal. In addition, it has a more important role, particularly for pathologists, as it allows researchers to identify the cell type and exact localization of the immunopositive. Hematoxylin is the most commonly used counterstain, although various colors are now being used as techniques of multiplex IHC progress.22 In the process of multiplex IHC,
counterstain should be selected carefully. The most commonly used counterstains are shown in Table 3.

**Troubleshooting**

There are many possible causes for poor staining results, which can be any of the following: weak or absent staining, unwanted background staining, or artifactual staining. Table 4 shows possible situations and solutions that are useful in troubleshooting.23

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**Table 3. Examples of counterstainings that are commonly used**

| Counterstain                  | Color   | Location   | Use                                                                 |
|------------------------------|---------|------------|---------------------------------------------------------------------|
| Hematoxylin (4 types: Harris’s, Mayer’s, Carazzi’s, and Gill’s) | Blue    | Nucleus    | The most popular one                                                 |
| Eosin                        | Red     | Cationic group of protein | Eosin is bound by the majority of structures in any tissue |
| Methylene blue               | Blue    | Nucleus    | Good to differentiate between DNA and RNA in tissues                |
| Methylene green              | Blue/green | Nucleus         |                                                                      |
| Toluidine blue               | Deep blue | Nucleus      | It will also stain polysaccharides a pink/red color (metachromasia) |

Toluidine blue stains melanin in green so that brown color of diaminobenzidine can be differentiated.

**Table 4. Consideration of variable troubleshootings and solutions**

| Problem                                      | Solution                                                                 |
|----------------------------------------------|--------------------------------------------------------------------------|
| Weak or absent staining                      |                                                                          |
| Antigen levels are too low                   | Prolong incubation time of primary antibody                               |
|                                              | Use a higher sensitivity staining system                                   |
| Incomplete fixation                          | Prevent under (> 30 min) or overfixation (> 48 hr)                        |
| Use of inappropriate fixative                | Check manufacturer’s specifications regarding recommended fixative       |
| Insufficient dehydration                     | Operating regular reagent changes (i.e., alcohol)                         |
| Paraffin too hot                             | Monitor temperature of paraffin (< 60°C)                                 |
| Embedding and dewaxing at high oven temperature | Oven temperature not to exceed 60°C                                       |
| Heating for antigen retrieval                | Optimize antigen retrieval time                                          |
| Reagents not working, reagents in wrong order | Monitor expiration dates, storage parameters, and pH                     |
| Antibody too dilute, improper antibody dilution | Determine correct concentration                                          |
| Partial drying out of tissue during processing | Immersing tissue immediately in fixative                                 |
|                                              | Use a humidity or moist chamber during incubation steps                   |
|                                              | Avoid evaporation with humidity chamber                                   |
| Chromogen not working, incorrect preparation of chromogen | Add chromogen to labeling solution                                      |
|                                              | Monitor for change in color                                              |
|                                              |                                                                          |
| Background or artifactual staining           |                                                                          |
| Excessive incubation                         | Reduce incubation time                                                   |
| Necrotic or otherwise damaged tissue         | Avoid sampling of necrotic areas                                          |
|                                              | Make sure tissue is properly fixed                                        |
| Antigen diffusion before fixation leading to specific background | Avoid delays in fixation                                                 |
| Thick preparation                            | Cut sections at 4 to 6 mm                                                 |
| Inappropriately concentrated antibody        | Check titration and concentration                                         |
|                                              | Decrease temperature of reaction                                         |
| Presence of chromogen or undissolved counterstain deposits | Filter the chromogen or counterstain                                     |
|                                              | Insure that chromogen is completely dissolved                             |
| Incomplete inadequate rinsing of slides      | Follow protocol for proper slide rinsing                                  |
|                                              | Mildy rinse slide with wash buffer bottle and place in wash bath in 5 min |
| Endogenous pigments                         | Check the negative control for the presence of these pigments            |
|                                              | Use a chromogen of contrasting color                                     |

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**QUANTIFICATION OF THE DATA**

To analyze the IHC data, including diagnostic value of certain molecular expression and prognostic relationship of the biomarkers, results of the IHC should be expressed in numerical values for statistical analysis. In this section, several quantification methods that are widely used are reviewed.

**Assessment of the proportion of immunopositive cells**

In evaluating IHC results, researchers commonly assess the
relative percentage of immunopositive cells in relation to the total number of target cells. Each value is usually recorded as a numerical score in every 10% (0, 0%–9%; 1, 10%–19%; 2, 20%–29%; 3, 30%–39%; 4, 40%–49%; 5, 50%–59%; 6, 60%–69%; 7, 70%–79%; 8, 80%–89%; and 9, 90%–100%). This method has a limitation that it does not show the intensity of the IHC staining, resulting in the lack of information regarding the subtle difference in protein expression level. However, it can preclude the interpretational error resulting from inappropriate variation in staining intensity between cases due to differences in tissue status in a large-scale study and among applied batches especially in manual staining.

Combinative semiquantitative scoring

Combinative semiquantitative scoring is the most commonly used method in the current prognostic biomarker researches, yielding immunoscores that incorporate both quantitative and qualitative assessments. In addition to the quantitative data from the assessment of relative percentage of immunopositive cells as previously described, intensity of the staining is also evaluated. The intensity is commonly scored from 0 to 3 (0, negative; 1+, weak positive; 2+, moderate positive; and 3+, strong positive). The final immunoscore is calculated by adding or multiplying each score. The Allred score used in breast cancer is one of the best-known combinative scoring systems.24 Although researchers can evaluate the IHC results both quantitatively and qualitatively with this method, too many variations can be created according to the combinations, resulting in different interpretations between researchers. Four or five score levels in average are recommended for the best sensitivity and reproducibility of the scoring system.25,26

Quantification using spectral image analysis

As multiplex IHC develops, analysis for multicolor stained specimen is essential. Usual semiquantitative scoring is not appropriate in the analysis of multiplex IHC due to a massive amount of information in a single slide. Thus, the use of spectral image analysis is increasing for the interpretation of multiplex IHC. In spectral unmixing, the optical signal from each chromogen can be isolated and assessed separately and quantitatively.18

ANALYSIS OF THE DATA

After quantitative analysis of the IHC results, the continuous variables are usually changed into categorical forms, as it is much easier to analyze and make decisions.20 The categorization of variables also enables clinicians to stratify patients according to the IHC results of the testing molecules. Cut-off values for immunopositivity are commonly selected by the median and the quartiles of measurements. However, sometimes simply more than 5% or 10% criteria are used. There is no standardized method for setting the cut-off value for the categorization. This lack of standardization sometimes causes inconsistent results between similar studies. Pathologists should be cautious when comparing IHC data between studies. To resolve these problems, there have been many efforts to establish reasonable ways to determine cut-off points. In this section, we introduce two statistical methods that are being frequently used.

Minimum p-value approaches (maximally selected chi-square statistics)

By this method, investigators can search cut-off points in a systematic manner. It means that all measured values are analyzed
as provisional cut-offs at first. Afterward, the value that has a maximum chi-squared statistic, minimum p-value, or maximum relative risk is selected. However, this method has a demerit of multiple testing. The p-value obtained from this method should be adjusted to offset the effect of multiple testing. In addition, specific statistics software such as SAS (SAS Institute Inc., Cary, NC, USA) or R software is required for this method.

Application of receiver operating characteristic curves

Receiver operating characteristic (ROC) curve analysis is another useful method for evaluating the prognostic relevance of biomarkers. ROC curves have been widely used in clinical oncology for evaluation and comparison of the sensitivity and specificity of the diagnostic tests regarding the binary outcomes. Recently, it has been increasingly used in the field of pathology research to determine cut-off scores in many cancers.

The ROC curve was initially developed to display signal-to-noise ratios. Basically, it is a plot of the ratio between the true positivity (sensitivity) and the false-positivity (1–sensitivity) (Fig. 2). In the ideal test, the ROC curve meets the upper left-hand corner. The diagonal reference line represents whether a test is positive or negative by chance. Performance of the test is quantified as a value of the area under the curve (AUROC). The better performance the test has, the closer to 1 the value of the AUROC is. Researchers can deduce specific cut-offs using various statistical methods. There are some pitfalls. Firstly, ROC curve analysis has to be applied in a test that has a gold standard. If the gold standard is uncertain, the entire interpretation of the results can become dubious.

Secondly, the cut-offs drawn from the ROC curves do not consider time or censoring of the data. Simply dichotomizing as “alive/censored” or “death” regardless of the follow-up time can be suboptimal in the prognostic research. To overcome this shortcoming, additional tests such as Kaplan-Meier survival analysis can be applied.

CONCLUSION

IHC has become an indispensable tool for pathologists in both everyday practice and basic research for elucidating pathophysiology of the diseases. In conjunction with this, IHC is also an indispensable tool for validation in biomarker discovery that will eventually lead to a personalized medicine. Even though IHC procedure has recently been automated and standardized, there are many things to be considered to optimize IHC properly and interpret appropriately. Optimization of IHC is particularly important for newly discovered molecules or new antibodies. Specificity and sensitivity of the IHC need to be validated. It is strongly recommended to review a full literature of the target molecule before starting IHC experiment. Interpretation of IHC also needs to be carefully planned. Consideration for stabilizing interobserver consistency and objectifying interpretation of IHC results is crucial. In this review, we attempted to provide basic principles and practical tips for practicing pathologists and residents in pathology training.

Conflicts of Interest

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

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The Predictive Value of Pathologic Features in Pituitary Adenoma and Correlation with Pituitary Adenoma Recurrence

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Background: The 2004 World Health Organization classification introduced atypical pituitary adenoma (aPA), which was equivocally defined as invasion with increased mitotic activity that had a Ki-67 labeling index (LI) greater than 3%, and extensive p53 immunoreactivity. However, aPAs that exhibit all of these features are rare and the predictive value for recurrence in pituitary adenomas (PAs) remains uncertain. Thus, we sought to characterize pathological features of PAs that correlated with recurrence. Methods: One hundred and sixty-seven cases of surgically resected PA or aPA were retrieved from 2011 to 2013 in Seoul St. Mary's Hospital. Among them, 28 cases were confirmed to be recurrent, based on pathologic or radiologic examination. The pathologic characteristics including mitosis, invasion, Ki-67 LI and p53 immunoreactivity were analyzed in relation to recurrence. Results: Analysis of the pathologic features indicated that only Ki-67 LI over 3% was significantly associated with tumor recurrence (p = .02). The cases with at least one pathologic feature showed significantly higher recurrence rates (p < .01). Analysis indicated that cases with two pathologic features, Ki-67 LI over 3% and extensive p53 immunoreactivity 20% or more, were significantly associated with tumor recurrence (p < .01). Conclusions: Based on these results, PA tumor recurrence can be predicted by using mitosis, invasion, Ki-67 LI (3%), or extensive p53 immunoreactivity (≥ 20%). Assessment of these features is recommended for PA diagnosis for more accurate prediction of recurrence.

Key Words: Pituitary neoplasms; Recurrence; Ki-67 antigen; Tumor suppressor protein p53

Pituitary adenomas (PAs) are defined as adenomas that are derived from adenohypophyseal cells in the sella turcica. PAs account for more than 10% of all intracranial tumors and are relatively common. Clinically, PAs are classified into nonfunctioning and functioning. They are divided into groups based on size, which include microadenomas (< 1 cm), macroadenomas (≥ 1 cm and < 4 cm), and giant adenomas (≥ 4 cm). Based on pathology, classification in the past was divided into three types (acidophilic, basophilic, and chromophobic), which was based only on morphologic features. However, they have recently been categorized into more fragmented classifications. The following characteristics are associated with clinically aggressive behavior: densely granulated prolactin-secreting adenomas, sparsely granulated monohormonal growth hormone-secreting adenoma, acidophilic stem cells, plurihormonal growth hormone-secreting adenoma, Crome's cell adenoma (aggressive variant of adenocorticotrophic hormone–secreting adenoma), sparsely granulated adenocorticotrophic hormone–secreting adenoma, thyroid-stimulating hormone-secreting adenoma, Null-cell adenoma (oncocytoma), and silent subtype 3 tumor. Evaluation of prognosis is limited, due to a discrepancy between immunohistochemically detected (produced) and secreted hormones.

Apart from the subtypes above, invasion into sinuses or recurrence after follow-up have often been identified by radiologic imaging techniques, such as magnetic resonance imaging. In 2004, the World Health Organization (WHO) designated a new group of atypical pituitary adenomas (aPA; International Classification of Diseases for Oncology [ICD-O] code 1). The aPA is defined by some markers that reflect the aggressive potential of PA. According to the WHO, aPA is a PA with aggressive features, such as invasion, and the diagnostic criteria include increased mitotic activity, Ki-67 labeling index (LI) > 3%, and extensive p53 immunoreactivity. Pituitary carcinomas (PC) with poor prognosis (4-year mean survival) can be diagnosed when craniospinal or systemic metastasis is confirmed. The aggressive features, however, are not well-defined and can have different interpretations, depending on the individual investigators. For example, Zada et al. excluded invasion from the criteria and...
reported 14.8% as a prevalence of aPA. However, when Saeger et al. applied criteria, including invasion, they reported a prevalence of 2.7%. In daily practice, the aPAs that correspond to all four features are actually very rare; therefore, the predictive value of these criteria for recurrence is questionable. Moreover, a recurrence has been reported in up to 36% of typical PAs.8-14 Aside from Ki-67 LI, definite cut-offs for increased mitotic activity and extensive p53 immunoreactivity have not been described in detail.1

The purpose of this study was to investigate the association between current aPA diagnostic criteria and tumor recurrence and to characterize the pathological features of PAs in order to establish more accurate prediction of tumor recurrence.

MATERIALS AND METHODS

The Institutional Review Board of the Catholic Medical Center approved this study (IRB No. KC14S1SI0833). From 2011 to 2013 in Seoul St. Mary’s Hospital, surgically-excised endocrine pituitary tumors were reviewed by two pathologists. One hundred and sixty-seven cases of pituitary tumors that were sequentially enrolled were identified. Only one aPA was included in these cases. Histologic features, such as increased mitotic activity and microscopic invasion (dura, bone, sinus, etc.), were assessed in relation to recurrence. The recurrence was defined as a newly-found mass that was diagnosed pathologically or radiologically identified. Immunohistochemistry analysis was performed with Ki-67 (ready-to-use, Roche, Indianapolis, IN, USA) and p53 (ready-to-use, Roche). Clinical data review was used to determine age, gender, residual mass, tumor size (< 1 cm, microadenoma; ≥ 1 cm and < 4 cm, macroadenoma; ≥ 4 cm, giant adenoma) and recurrence after operation.

The following criteria were used to define aPA: (1) invasion, (2) a Ki-67 LI greater than 3%, (3) extensive p53 immunoreactivity, and (4) increased mitotic activity. PC were diagnosed by identifying distant metastases.8 Invasion into adjacent dura, bone and/or surrounding anatomical structures were taken into account. We manually counted 1,000 cells in the hot spot area for Ki-67 LI. Extensive p53 immunoreactivity was defined as intensive nuclear reactivity of 20% or more in tumor cells and increased mitotic activity was defined as 2/10 high power field (HPF) or more. The four features and cut-offs listed above were analyzed in relation to recurrence, in combination with or without other characteristics as follows: invasion, Ki-67 LI > 3%, extensive p53 immunoreactivity,mitosis ≥ 2/10HPF, and any additional criteria, as listed above. Other characteristics such as gender, age at diagnosis, tumor size, presence of residual tumor and recurrence were also analyzed. For the qualitative variable comparison, student t test, Pearson’s chi-square test and linear by linear association were used. Every p-value used in this study was two-sided, and a value less than .05 (p < .05) was counted as statistically significant. Moreover, odds ratios (OR) were calculated. OR were used to measure the association between a variable and an outcome. The OR is defined as the probability of an outcome occurring with a specific variable, compared to the odds that the outcome will occur without that variable.15

Table 1. Clinical characteristics and recurrence rates

| Characteristic | All patients | Patients with recurrence | Patients without recurrence | p-value | Odds ratio |
|---------------|-------------|--------------------------|-----------------------------|---------|------------|
| No. (%)       | 167 (100)   | 28 (16.8)                | 139 (83.2)                  |         |            |
| Mean age (range) | 49 (19–80) | 43 (20–71)               | 50 (19–80)                  | .02*    | -          |
| Sex           |             |                          |                             | .61     |            |
| Male          | 79 (47.3)   | 12 (15.2)                | 67 (84.8)                   |         |            |
| Female        | 88 (52.7)   | 16 (18.2)                | 72 (81.8)                   |         |            |
| Tumor size*   |             |                          |                             | > .05   | -          |
| Microadenoma  | 15 (9)      | 2 (13.3)                 | 13 (86.7)                   |         |            |
| Macroadenoma  | 141 (84.4)  | 21 (14.9)                | 120 (85.1)                  |         |            |
| Giant adenoma | 11 (6.6)    | 5 (45.5)                 | 6 (54.5)                    | < .01*  | 4.85       |
| Residual mass |             |                          |                             | < .01*  | 6.27       |
| Absent        | 118 (70.7)  | 10 (8.5)                 | 105 (89)                    |         |            |
| Present       | 49 (29.3)   | 18 (36.7)                | 31 (63.3)                   |         |            |

Values are presented as number (%).
*Significant difference (p < .05).
*Classified into microadenoma (< 1 cm), macroadenoma (≥ 1 cm and < 4 cm), giant adenoma (≥ 4 cm).

http://jpatholtm.org/
statistical software ver. 21.0 (IBM Co., Armonk, NY, USA) was used for statistical analyses.

RESULTS

The clinical characteristics and recurrence rates are summarized in Table 1. Twenty-eight out of 167 patients (16.8%) experienced recurrence. The mean age at first diagnosis in the group with recurrence was 43 years which was significantly younger than that of the group without recurrence, which was 50 years (p = .02). Although there were more female patients than male patients, the difference was not statistically significant (p = .61). Recurrence rates were 13.3% in microadenoma, 14.9% in macroadenoma, and 45.5% in giant adenoma. The analysis indicated that a higher recurrence rate was associated with larger tumor size, although this result was not statistically significant (p > .05). Analysis of the giant adenomas in relation to recurrence indicated that there was a significant association with recurrence (p = .01; OR, 4.85). The presence of residual mass after surgery was observed in 49 patients (29.3%). In the group with residual mass, 18 patients (36.7%) experienced recurrence, which was significantly higher than recurrence (8.5%) in the group without residual mass (p < .01; OR, 6.27).

Reclassification of the study population using histopathologic features with recurrence rates are summarized in Tables 2–4. In the group with recurrence, the mean Ki-67 LI was 4.04% and was greater than that of the group without recurrence, which was 1.95%; however, this result was not significantly different (p = .14). The Ki-67 LI over 3% was significantly associated with recurrence (p = .02; OR, 2.99). In regard to invasion, the

| Characteristic | Patients with recurrence (n = 28) | Patients without recurrence (n = 139) | p-value | Odds ratio |
|---------------|----------------------------------|---------------------------------------|---------|------------|
| Ki-67, mean   | 4.04                             | 1.95                                  | .14     |            |
| Labeling index (≥ 3%) | 9 (32.1) | 19 (67.9) | .02* | 2.99 |
| Invasion      | 9 (32.1)                         | 19 (67.9)                             | .14     |            |
| p53 immunoreactivity (≥ 20%) | 2 (7.1) | 26 (92.9) | .16     |            |
| Mitosis, mean | 1.11                             | .26                                   | .31     |            |
| ≥2/10 HPF     | 3 (10.7)                         | 25 (89.3)                             | .06     |            |

Values are presented as number (%).
*Significant difference (p < .05).

| Combination of criteria | Patients with recurrence (n = 28) | Patients without recurrence (n = 139) | p-value | Odds ratio |
|-------------------------|----------------------------------|---------------------------------------|---------|------------|
| I/K3/p20/M2             |                                  |                                       |         |            |
| ≥1                      | 17 (60.7)                        | 11 (39.3)                             | < .01*  | 3.96       |
| ≥2                      | 4 (14.3)                         | 24 (85.7)                             | .51     |            |
| All (4)                 | 1 (3.6)                          | 27 (96.4)                             | .03*    |            |

I, invasion; K3, Ki-67 LI > 3%; p20, p53 immunoreactivity ≥ 20%; M2, mitosis ≥2/10 HPF.
*Significant difference (p < .05).

| Combination of criteria | Patients with recurrence (n = 28) | Patients without recurrence (n = 139) | p-value | Odds ratio |
|-------------------------|----------------------------------|---------------------------------------|---------|------------|
| K3 and I                |                                  |                                       | .43     |            |
| K3 and p20              |                                  |                                       | .01*    |            |
| K3 and M2               |                                  |                                       | .44     |            |
| I and p20               |                                  |                                       | .44     |            |
| I and M2                |                                  |                                       | .21     |            |
| p20 and M2              |                                  |                                       | .03*    |            |

K3, Ki-67 LI > 3%; I, invasion; p20, p53 immunoreactivity ≥ 20%; M2, mitosis ≥2/10 HPF.
*Significant difference (p < .05).
The recurrence rate was slightly higher in cases with invasion (32.1%) than in cases without invasion (19.4%); however, this result was not significant (p = .14). Only five patients showed extensive p53 immunoreactivity and two of them experienced recurrence (p = .16). We assessed the relationship between increased mitotic activity and recurrence but did not identify any significant differences, and in most cases, mitosis with 1/10HPF or less was observed. The mean mitotic count was 1.11/10HPF in the group with recurrence versus 0.26/10HPF in the group without recurrence (p = .31) (Table 2). There was a significant correlation between the group that satisfied at least one of the four identified features and recurrence (p < .01; OR, 3.96). The cases with two or more features were not significantly correlated with the results (p = .51) (Table 3). Among the cases with two specific features, the group with Ki-67 LI > 3% and extensive p53 immunoreactivity experienced statistically significant recurrence (p < .01). The group with extensive p53 immunoreactivity and increased mitotic activity had significant recurrence; however, only one of these cases was identified (p = .03). The other relationships were analyzed; however, no statistically significant relationship was identified (Table 4). Only one aPA patient met all four of the WHO criteria (Fig. 1), and the only case which satisfied any 3 or 4 of the criteria was determined to have a statistically significant relationship with recurrence (p = .03) (Table 3). Approximately 1 year from the first diagnosis, this case was confirmed be PC after the metastasis was identified to the distant cerebrum and skull (Fig. 2).

**DISCUSSION**

In this present study, analyzing cases with at least one of the diagnostic criteria contributed to predicting recurrence. This result indicates that recurrence would be significantly lower in patients that do not show any of the aPA criteria, compared
Predictive Value in Pituitary Adenoma

Among the four criteria, cases with Ki-67 LI > 3% alone or along with extensive p53 immunoreactivity showed similar results.

Although many predictive factors have been reported to be related with tumor recurrence in PAs, there is controversy about whether these factors are practically useful. The WHO’s diagnostic criteria for aPA is poorly defined and its application is currently under discussion.9,16-20 There is no definite cut-off for extensive p53 immunoreactivity and increased mitotic activity. The features in the criteria are also frequently observed in PA. We found that a valid cut-off for Ki-67 LI is > 3%. Matsuyama21 and Lee et al.10 compared Ki-67 LI between patients with and without recurrence and found that it was significantly different. However, Sadeghipour et al.11 and Hadzhiyanev et al.12 found no significant result when they compared mean Ki-67 LI between patients with recurrence and those without recurrence. Many studies have compared PA and aPA. Miermeister et al.22 defined the inclusion criteria of aPA as a minimum three of criteria and reported Ki-67 LI > 4% as the best marker to discriminate between PA and aPA. In our study, however, Ki-67 LI > 4% showed no significant difference in relation to recurrence (p = .28). We analyzed Ki-67 LI in the group with recurrence, and the mean value was 4.04 and the standard deviation was 7.24. Five cases with Ki-67 LI > 3% but ≤ 4% were found in the group with recurrence (n = 28).

We concluded that invasion alone was not a significant factor for predicting recurrence. Similarly, Matsuyama21 found that suprasellar extension and/or cavernous sinus invasion were not meaningfully associated with recurrence. Several studies comparing PA and aPA showed significant results.9,13,14,22 Specifically, Chiloiro et al.14 reported that the cases with cavernous invasion were associated with significant results; however, the cases with the other characteristics, including suprasellar extension, were not significant. Our results could be limited because we did not
classify cases by the location of the invasion.

In this study, increased mitotic activity was not associated with recurrence. Miermeister et al. suggested the cut-off value of mitotic count 2/10HPF or more as the best criterion to discriminate between PA and aPA. When applying the criterion in this study, there was no significant relationship between recurrence and increased mitotic activity. Moreover, when comparing the mean mitotic count between the groups with and without recurrence, there was no significant difference. In comparison, Lee et al. suggested the cutoff value of mitotic count 1/10HPF or more as a good predictive marker for recurrence. We also applied this criterion in our study, although there was no meaningful association between increased mitosis (1/10HPF or more) and high recurrence rate (data not shown).

Analysis of extensive p53 immunoreactivity did not indicate any significant results. Similarly, Hadzhiyanev et al. compared p53 nuclear positivity (at least weak or focal expression) and did not identify any significant difference. However, Lee et al. compared p53 intensive nuclear activity ≥ 3% between patients with and without recurrence and reported significant results. Miermeister et al. suggested p53 intensive nuclear activity ≥ 2% as the best criterion for aPA.

Cases with Ki-67 LI > 3% along with extensive p53 immunoreactivity were significantly associated with recurrence (p < .01). Invasion with extensive p53 immunoreactivity, and invasion with increased mitotic activity, were not significantly associated with recurrence, with the exception of combinations with specific two features, Ki-67 LI > 3% with invasion and Ki-67 LI > 3% with increased mitotic activity. Although there was a statistical association with recurrence in the group with extensive p53 immunoreactivity, and increased mitotic activity (p = .03), a careful interpretation is needed because there was only one case which met this criteria. Although it can be assumed that the statistical significance of Ki-67 LI > 3% affected the result to some degree, pairing Ki-67 LI > 3% with one of the other factors, except extensive p53 immunoreactivity, was not associated with significant differences.

The mean age of the group with recurrence was significantly younger than the other group and Matsuyama reported that there was no significant relationship between recurrence and the giant tumors, but Lee et al. reported that tumors which measured ≥ 25 mm significantly recurred more frequently than tumors that were < 25 mm. Tortosa and Webb found that tumors that were ≥ 1 cm were identified significantly more frequently in aPA than PA. In spite of the same cut-off for size as Tortosa and Webb, Zada et al., and Chiloiro et al. found no significant differences.

The group with residual masses after surgery showed significantly more recurrence and Matsuyama reported similar results. However, these results were different from those reported by Lee et al. According to Chiloiro et al., there was no significant difference between PA and aPA. Because residual mass assessment was performed by gross examination at surgery or radiologic methods with controversial criteria, differentiating residual mass after excision and true tumor recurrence is limited.

There were some limitations to this study: (1) the number of cases retrieved was relatively small, (2) the possibility of radiologic inaccuracy in the assessment of inversion or residual mass after surgery, and (3) no definite standard for extensive p53 immunoreactivity and increased mitotic activity, such as cut-off or intensity. We investigated the predictive value of clinical and pathologic characteristics in relation to recurrence. Even though PA cases did not meet all the criteria, using any single pathologic feature can be useful to predict recurrence. Ki-67 LI with a cut-off > 3% with or without extensive p53 immunoreactivity (≥ 20%) was significantly associated with recurrence. Assessment of these features can be applied in PA diagnosis for more accurate prediction of recurrence. Additional studies with a larger sample population will be important for identifying more detailed, reproducible and clear features for markers that can be used to predict recurrence.

**Conflicts of Interest**

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

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Morphological and Functional Changes in the Thyroid Follicles of the Aged Murine and Humans

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Background: Although both thyroid histology and serum concentrations of hormones are known to change with age, only a few reports exist on the relationship between the age-related structural and functional changes of the thyroid follicles in both mice and humans. Our objectives were to investigate age-related histological changes of the thyroid follicles and to determine whether these morphological changes were associated with the functional activity of the follicles. Methods: The thyroid glands of mice at 18 weeks and at 6, 15, and 30 months of age were histologically examined, and the serum levels of thyroid hormones were measured in 11-week-old and 20-month-old mice. Samples of human thyroid tissue from 10 women over 70 years old and 10 women between 30 and 50 years of age were analyzed in conjunction with serum thyroid hormone level. Results: The histological and functional changes observed in the thyroid follicles of aged mice and women were as follows: variable sizing and enlargement of the follicles; increased irregularity of follicles; Sanderson’s polsters in the wall of large follicles; a large thyroglobulin (Tg) globule or numerous small fragmented Tg globules in follicular lumens; oncocytic change in follicular cells; and markedly dilated follicles empty of colloid. Serum T3 levels in 20-month-old mice and humans were unremarkable. Conclusions: Thyroid follicles of aged mice and women show characteristic morphological changes, such as cystic atrophy, empty colloid, and Tg globules.

Key Words: Aged; Elderly; Thyroid gland; Thyroid hormones

The functional unit of the thyroid gland is the thyroid follicle, which is composed of follicular cells and intrafollicular colloid. The size and shape of thyroid follicles and the height of the follicular epithelium vary depending on the thyroid’s functional activity. In addition, there is morphological heterogeneity of the intrafollicular colloid depending on the thyroid’s functional status.1

Aging induces morphological and functional changes in the thyroid and leads to gradual loss of the ability to maintain homeostasis. Increases in size and number of follicles have been reported in the aged male albino rat and in the humped camel;2 however, the thyroid of a human over the age of 60 undergoes progressive fibrosis and atrophy, leading to a reduction in thyroid volume.3,4

The loss of follicular cells due to age-associated cell death has been reported, but the loss of thyroid function is debated.

Most pronounced age-related changes occur in nondividing and infrequently dividing cells that have longer turnover times, such as brain (turnover time of neurons, about 16,425 days), muscle (turnover time of myocyte, about 5,510 days), and liver (turnover time of hepatocyte, about 327 days). Longer cell turnover time allows for a greater accumulation of DNA damage with age. Cells that accumulate DNA damage may have reduced viability and loss of function, which eventually lead to tissue atrophy. Human thyroid follicular cells have a longer turnover time (about 3,180 days) than cells of other endocrine organs, such as the adrenal gland (about 455 days) and pancreas (about 265 days).5

Although there are some studies on age-related histological changes of the thyroid gland, there are few on the relationship between histological changes and functional activity of aged thyroid follicles. The present study examines age-related structural and functional changes in the thyroid follicles and investigates the impact of these changes on serum thyroid hormone concentrations.
MATERIALS AND METHODS

Animals and tissue histology

Thyroids were excised from C57BL/6 male mice that were sacrificed at 18 weeks (n = 2), 6 months (n = 2), 15 months (n = 2), or 30 months (n = 2) of age. The mice had been fed a normal chow diet since birth and were maintained in accordance with the principles of laboratory animal care. For analysis, they were grouped as follows: control mice (18 weeks old), adult mice (six months old), and aged mice (15 and 30 months old). Blood was collected from the retro-orbital sinus in anesthetized 11-week-old (n = 7) or 20-month-old (n = 7) C57BL/6 male mice in order to measure serum thyroid hormone levels. The mice were subsequently sacrificed.

Each thyroid was fixed in 10% neutral buffered formalin and paraffin-embedded in the transverse plane using standard procedures. Paraffin-embedded tissue sections (4-μm-thick) were stained with hematoxylin and eosin (H&E) for histological analysis, which included follicle size and shape, follicular cell height, and characteristics of the cytoplasm and intrafollicular colloid.

Tissue sections were stained with periodic-acid-Schiff (PAS), which stains the glycoprotein thyroglobulin (Tg) in the colloid purple-red. The PAS stain intensity of the intrafollicular colloid was then compared between samples. In hypoactive follicles, Tg accumulates in the colloid and stains dark blue-purple with PAS.

All mouse experiments were approved by the university committee for animal experiments and were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and in accordance with the Guidelines for the Care and Use of Laboratory Animals prepared by the Institute for Laboratory Animal Research, National Academy of Sciences.

Measurements of thyroid hormone

Retro-orbitally collected, clotted mouse blood was centrifuged at 3,000 ×g for 10 minutes. Sera were separated and stored at −20°C prior to the hormonal assay. Total T3 and T4 levels were measured using an enzyme-linked immunosorbent assay kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer’s instructions. Serum thyroid-stimulating hormone (TSH) was measured using a specific mouse TSH radioimmunoassay provided by Dr. Cheng S.Y. (Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA).

Analysis of size and shape of thyroid follicles

Sections of the whole thyroid gland (approximately 20 serial sections) at ×100 magnification were used to assess the area of the follicles. The inner area of the thyroid gland at half the maximum length in the longitudinal planes was considered the central zone, and the area surrounding the central zone was considered the peripheral zone (Fig. 1A, left). The same approach was taken in the transverse planes of the thyroid gland (Fig. 1A, right). The size of the follicles in the central zone was compared with the size of the follicles in the peripheral zone.

Follicles that were neither ovoid/round nor smooth in outline were considered to be of irregular shape. Irregularly-shaped follicles were assessed in 20 serial sections using ×100 magnification images of the whole thyroid gland section.

Fig. 1. The 18-week-old control thyroid gland. (A) Description of the central and peripheral zones of the thyroid gland in the longitudinal and transverse planes. (B, C) Conserved zonal variation in the thyroids of 18-week-old control mice. (B) Hematoxylin and eosin-stained sections of thyroid from an 18-week-old control mouse in the central and peripheral zones. (C) Mean area (± standard deviation, n = 2) of follicles in the central and peripheral (pph) zones of an 18-week-old mouse thyroid.
Immunohistochemistry analysis

To assess mitochondrial content, tissue sections were immuno- 
stained with an antibody against the translocase of the outer 
mitochondrial membrane (TOM20). Paraffin-embedded tissue 
sections (4-μm-thick) were placed in an oven and incubated at 
56°C for three hours before immunohistochemistry. Specimens 
were stained using the Ventana HX automatic system from 
BenchMark (Ventana Medical Systems, SA, Illkirch Cedex, 
France) with an anti-TOM20 rabbit polyclonal antibody (Santa 
Cruz Biotechnology, Santa Cruz, CA, USA). All procedures, 
including antigen retrieval and blocking of endogenous peroxi-
dase activity, were performed automatically by the BenchMark 
system. The tissue sections were incubated with primary antibody 
for 32 minutes at 42°C. Immunoperoxidase staining was per-
formed using the LSAB NeuVision system, according to the 
manufacturer’s instructions (Ventana Medical Systems), and 
sections were counterstained with hematoxylin. Tissue slides 
were analyzed using an OLYMPUS BX51 microscope (Olympus, 
Tokyo, Japan).

The immunoreactivities of TOM20 were analyzed for all cases. 
The intensity of TOM20 immunoeexpression was scored as follows: 
0, negative; 1, weakly positive; 2, moderately positive; and 3, 
strongly positive.

Human tissue histology and clinical data

H&E-stained, paraffin-embedded thyroid tissue and thyroid 
function test results (serum concentrations of TSH, T3, and T4) 
from 10 women over the age of 70 and 10 women between 30 
and 50 years of age who underwent total thyroidectomy between 
January 2002 and December 2005 at Daejeon St. Mary’s Hospital, 
Daejeon, Korea, were analyzed retrospectively. All patients pre-
sented with papillary thyroid carcinoma (PTC). Normal thyroid 
tissues of the contralateral lobe of PTC were analyzed for age-
related change. The study protocol was reviewed and approved 
by the Institutional Review Board of Daejeon St. Mary’s Hospital, 
College of Medicine, The Catholic University of Korea. All partic-
ipants provided signed, written informed consent. All exper-
iments were performed in accordance with relevant guidelines 
and regulations. The baseline characteristics and thyroid function 
test results of each human participant are summarized in Table 1.

Statistical analysis

Group comparisons of categorical variables were evaluated 
using a linear-by-linear association. The means were compared

| Table 1. Summary of human characteristics and thyroid function tests |
|-------------------|-----------------|-----------------|-----------------|
| Age (yr)          | Serum T3 (nmol/L) | Serum T4 (µg/dL) | TSH (uIU/mL)    |
| 30–50-year-old women |
| 36                | 1.3             | 8.5             | 1.42            |
| 39                | 1.1             | 7.63            | 1.53            |
| 44                | 1.0             | 8.2             | 2.03            |
| 45                | 1.07            | 9.08            | 2.53            |
| 46                | 1.2             | 12.1            | 2.67            |
| 46                | 1.0             | 7.8             | 2.57            |
| 46                | 1.0             | 9.5             | 2.6             |
| 47                | 1.1             | 6.1             | 5.28            |
| 47                | 1.1             | 6.35            | 3.48            |
| 50                | 1.0             | 8.6             | 1.15            |
| Mean ± SD         | 1.087 ± 0.096   | 8.386 ± 1.696   | 2.526 ± 1.196   |
| > 70-year-old women |
| 71                | 1.3             | 7.5             | 2.86            |
| 71                | 1.4             | 7.3             | 0.32            |
| 73                | 1.2             | 4               | 2.37            |
| 74                | 1.9             | 7.76            | 0.01            |
| 75                | 1.3             | 7.3             | 3.83            |
| 75                | 1.38            | 7.3             | 1.74            |
| 77                | 1.61            | 7.3             | 1.9             |
| 77                | 1.2             | 8.8             | 2.54            |
| 77                | 1.1             | 8.4             | 0.16            |
| 78                | 1.09            | 7.3             | 0.75            |
| Mean ± SD         | 1.348 ± 0.248   | 7.296 ± 1.274   | 1.648 ± 1.296   |

Normal range: TSH, 0.27–4.2 uIU/mL; serum T3, 1.3–3.1 nmol/L; serum T4, 5.4–11.5 µg/dL; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone; SD, standard deviation.
with the result of the independent sample t-test. Values are represented as the mean ± SEM. A p-value less than .05 was considered statistically significant. Analyses were performed using SPSS ver. 22.0 statistical software (IBM Corp., Armonk, NY, USA).

RESULTS

Serum thyroid hormone concentrations and thyroid functional activity in aged mice

We measured serum levels of TSH, T3, and T4 in 20-month-old mice (aged group; n = 7) and compared them with levels of the same hormones in 11-week-old mice (control group; n = 7) (Table 2). Serum T3 concentrations were lower in the aged group than in the control group, but there was no difference in serum T4 and serum TSH levels between the two groups.

The thyroid activation index, expressed as the ratio of follicular cell volume to colloid volume, reflects changes in thyroid function caused by alterations in TSH level.6,7 Thyroid tissue from 18-week-old control mice was composed of a relatively homogeneous population of small- to medium-sized round follicles lined with cuboidal epithelium (Fig. 1B). In contrast, the 30-month-old mouse thyroid was composed of dilated or irregularly-shaped follicles (Fig. 2E), and the dilated follicles were lined with flat epithelium. The intrafollicular colloid volume was larger in 30-month-old mice than in the controls. Consequently, the younger thyroid, which was composed of cuboidal or columnar epithelium and a small amount of colloid, had a higher thyroid activation index than the older thyroid, which was composed of a flat epithelium and significantly more colloid.

Irregularly enlarged thyroid follicles in aged mice

Next, we examined whether the histological changes in the thyroid follicles of aged mice correlated with thyroid function. The 18-week-old control thyroid glands were composed of small- to medium-sized round follicles. The larger follicles tended to localize in the peripheral region of the thyroid gland. These distributions of follicles is called the zonal variations in follicle size.8,9 Zonal variation in the size of thyroid follicles was well preserved. Thyroid follicles of the central zone were smaller than those of the peripheral zone, with a ratio of the area of central zone to peripheral zone follicles of 1:2 (Fig. 1B, C). The adult mouse thyroid gland (6 months old) was composed of variable-sized follicles with a relatively round shape. The zonal variation in the size of thyroid follicles was well preserved; however, follicles in the peripheral zone were three times larger than those in the central zone (Fig. 2A, B). Smaller follicles found in the central zone were lined with a cuboidal epithelium, and larger follicles located in the peripheral zone were lined with a cuboidal or low cuboidal epithelium (Fig. 2A). Thyroid glands from aged mice (15 months old and 30 months old) were composed of irregularly dilated follicles, which were considered to be inactive (Fig. 2C, E). There was a greater incidence of inactive large follicles in the aged mice compared to the thyroid of adult mice. Zonal variation in the size of thyroid follicles was not conserved (Fig. 2D, F). Dilated follicles were not only present in the periphery of the gland, but were also seen in the central zone. The aged thyroids were extremely heterogeneous in appearance, exhibiting variability in the height of the follicular epithelium and in the amount of intrafollicular colloid. Furthermore, papillary and glandular proliferations were observed in the epithelium (Fig. 3A, B). Irregularly-shaped follicles were also observed in aged mouse thyroid glands. This was more frequently observed in the 30-month than in the 15-month-old mice (Fig. 3A, B). Most irregularly-shaped follicles were surrounded by a cuboidal or high cuboidal epithelium.

In the thyroid glands of aged mice, some of the large dilated follicles had cellular areas composed of small follicles lined by a crowded epithelium with scant colloid in a small lumen; these were pushing into large colloid-filled follicles. This characteristic lesion is referred to as a Sanderson’s polster (Fig. 3A, B). Some of the large dilated follicles also had asymmetric pseudopapillary

Table 2. Mouse thyroid function tests

| No. | 11-week-old mice |  | 20-month-old mice |  |
|-----|------------------|---|------------------|---|
|     | Serum T3 (ng/mL) | Serum T4 (µg/dL) | TSH (ng/mL) | Serum T3 (ng/mL) | Serum T4 (µg/dL) | TSH (ng/mL) |
| 1   | 0.77             | 4.33                      | 139.36       | 0.23             | 3.84              | 29.45       |
| 2   | 0.86             | 3.51                      | 72.28        | 0.16             | 3.70              | 39.51       |
| 3   | 0.71             | 4.49                      | 82.22        | 0.09             | 3.35              | 135.39      |
| 4   | 0.90             | 4.07                      | 155.40       | 0.13             | 3.85              | 56.85       |
| 5   | 0.91             | 4.53                      | 50.75        | 0.25             | 4.46              | 93.22       |
| 6   | 0.66             | 3.77                      | 82.07        | 0.12             | 5.40              | 73.80       |
| 7   | 0.55             | 3.48                      | 79.56        | 0.33             | 5.41              | 52.10       |
| Mean ± SD | 0.766 ± 0.116 | 4.026 ± 0.386            | 94.52 ± 32.897 | 0.187 ± 0.074 | 4.287 ± 0.719 | 68.617 ± 31.391 |

T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone; SD, standard deviation.
projections of crowded columnar cells (Fig. 3B).

**Tg globules in the follicular lumen of aged mice**

Intrafollicular colloid of control and adult thyroids stained homogeneously pink by H&E (Figs. 1B, 2A). In the control group, many endocytic vacuoles were seen near the margin of the colloid-filled lumen (Fig. 1B, arrow). These vacuoles emerge with dissolution of colloid by acid phosphatase that is secreted from lysosomes in conjunction with hormonal release during follicular cell activity.\(^\text{10}\) Thus, colloid endocytosis correlates with follicular cell activity. In 6-month-old adult thyroids, several follicles in the peripheral zone were empty and were lined with a high cuboidal epithelium. The follicular cells of the empty follicles had clear cytoplasm and a centrally located, shrunken nucleus (Fig. 2A, arrow). These clear cell changes were suggestive of a distended endoplasmic reticulum, which was interpreted as a sign of follicular degeneration. Colloid vacuolation was frequently observed in the adult thyroid.

In the aged thyroid (15 months old and 30 months old), colloid-filled follicles lined with a flat epithelium stained homogeneously pink with H&E, while colloid within the follicles lined with a high cuboidal epithelium was pale and coarsely granular (Fig. 2C, E). There were numerous small, fragmented, and clumped Tg globules within the intrafollicular colloid that were surrounded by a high cuboidal or oncocytic epithelium (Figs. 2E, 3B). The formation of Tg multimers allows for storage of Tg at excessively high concentrations, and they are more frequently observed in hypofunctioning follicles.\(^\text{11}\) Some of the thyroid follicles were empty, and others contained only small amounts of colloid in their larger lumen (Fig. 2E). Empty follicles are inactive.
Fig. 3. Increased irregularity of follicles in the aged mouse thyroid. Hematoxylin and eosin (H&E)–stained sections of thyroid from 15-month-old (A) and 30-month-old (B) mice. (C) Oncocytic change in the follicular cells of aged mouse thyroid stained with H&E. (D) Sections of 30-month-old mouse thyroid stained with anti-translocase of the outer mitochondrial membrane (TOM20) antibody visualized by 3,3′-diaminobenzidine. Non-oncocytic follicular cells are weak for TOM20 (left panels), while oncocytic follicular cells are strongly TOM20-positive (right panels). Tg, thyroglobulin.
follicles that may have lost accumulated colloid over time due to stasis of colloid circulation. Colloid vacuolation, which signifies colloid resorption, was occasionally observed. Briefly, these findings indicated decreased thyroid gland activity with increasing age.

**Oncocytic change in thyroid follicular cells in aged mice**

In 30-month-old mouse thyroids, follicles lined with a low columnar epithelium showed oncocytic changes that were characterized by an abundant oxyphilic, granular cytoplasm and a large hyperchromatic nucleus (Fig. 3C). An oncocytic appearance is the result of mitochondrial proliferation, which is a mechanism to compensate for mitochondrial defects. Compared with non-oncocytic follicular cells, oncocytic follicular cells were intensely stained by the TOM20 antibody. The TOM20 score was 1 and 3 in non-oncocytic follicular cells and oncocytic follicular cells, respectively (Fig. 3D).

**Colloid densities in follicular lumens**

After analyzing the intensity and multimerization of PAS-stained intrafollicular colloid, we compared intrafollicular colloid concentrations and follicular activity for each age group. Fig. 4 shows the PAS-positive density of the colloid in each group.

Regular thyroid follicles of the adult group (6 months old) were filled with homogeneously PAS-positive colloid. PAS-positive colloid in the peripheral zone was more darkly stained than the PAS-positive colloid in the central zone. Few follicles exhibited PAS-negative lumens (Fig. 4A).

The markedly enlarged follicles of the aged group (15 months old and 30 months old) had an increased PAS-positive density (dark blue-purple PAS stain) (Fig. 4C). The colloid in irregularly-shaped follicles and in small follicles was stained purple-red by PAS. Sanderson’s polsters were observed on one side of the large, dilated follicles. These aggregated, small follicles are composed of a high cuboidal epithelium and lead to depletion of luminal colloid. Sanderson’s polsters were heterogeneously stained by PAS. Abundant colloid in large follicles was stained purple-red or dark blue-purple by PAS, while cellular areas composed of small follicles were pale-red or negative (Fig. 4C). Thus, the markedly enlarged follicles seen in aged mice were considered to be hypoactive compared with small follicles, irregularly-shaped follicles, and Sanderson’s polsters.

**Thyroid histology and thyroid function tests in elderly women**

We also analyzed age-related histological changes in the thyroid glands of 10 women over 70 years of age and compared them...
with thyroids from women between 30 and 50 years of age. We observed findings similar to those seen in aged mice, including (1) variable size and enlargement of follicles (Fig. 5A); (2) increased irregularity of the follicles (Fig. 5B); (3) Sanderson's polsters in the walls of large follicles (Fig. 5B); (4) a large Tg globule or numerous, small, fragmented Tg globules in the follicular lumen (Fig. 5C);

Fig. 5. Sections of thyroid from elderly females highlighting various morphological changes. (A) Size variation and enlargement of the follicles. (B) Increased irregularity of follicles and Sanderson’s polsters in the wall of large follicles. (C) A large thyroglobulin (Tg) globule (right upper image) and numerous small fragmented Tg globules (right lower image) in the follicular lumen. (D) Oncocytic change in the follicular cells and in those with Sanderson’s polsters. (E) Irregularly dilated follicles lacking colloid. (F) Fibrosis of the extracellular matrix. (G) Fatty infiltration in the extracellular matrix.
(5) oncocytic change in the follicular cells (Fig. 5D); and (6) markedly dilated follicles lacking colloid (Fig. 5E). However, unlike the thyroids of aged mice, fibrosis or fatty infiltration within the extracellular matrix was observed in elderly human thyroids (Fig. 5F, G).

Among the 10 elderly women, thyroid hormone levels were unremarkable when compared with controls (T3, p = .551; T4, p = .138; TSH, p = .085) (Table 1). Age-related histological changes and functional activities in the human thyroid follicles were not associated with changes in serum thyroid hormone levels.

**DISCUSSION**

In this study, we identified age-related histological changes in the thyroid glands of aged mice. These changes included a decrease in the entire thyroid size, formation of markedly dilated follicles with a flat epithelium, irregularly-shaped follicles, aggregations of small follicles with oncocytic epithelia, the presence of colloid-depleted follicles, a large Tg globule or multimeric Tg globules within the colloid, and loss of zonal variation. Fibrosis, inflammation, and fatty infiltration were common in elderly human thyroids but rarely observed in aged mouse thyroids. Of these changes, the height of the follicular epithelium, cytoplasmic features of follicular cells, PAS staining properties of the colloid, and characteristic Tg globules are representative of functional activity of the thyroid follicles. A commonly encountered pattern in the aged mice thyroid was prominent cystically dilated follicles with flimsy walls composed of scant fibrous stroma. We attributed this characteristic histological finding to cystic atrophy of the follicle. Cystic atrophy is not infrequently found in the postmenopausal endometrium. In the absence of ovarian function, the endometrium experiences cystic atrophy, having a thin uterine mucosa, cystically dilated endometrial glands, and a flattened inactive epithelium. The pathogenesis of cystic atrophy of the endometrium has not yet been established. In cystic atrophy of the aged thyroid, low cuboidal or flattened and inactive follicular cells line the distended follicles. Larger or dilated follicles lined with a low cuboidal epithelium contain colloid that stains purple-red with PAS, while larger or dilated follicles with a flat epithelium contain thick colloid that stains dark blue-purple with PAS. As organisms get older, the function of thyroid follicular cells tends to diminish, thus decreasing endocytosis of luminal colloid into follicular cells. Eventually, the accumulation of luminal colloid increases intrafollicular pressure and increases tension in the follicular wall, which may contribute to the flattening of follicular cells.

Oncocytic change in cells is increasingly observed with advancing age in thyroid glands and in other organs. In the invovled thyroids of old mice, a follicular epithelium with oncocytic change is more frequently observed. The characteristic oncocytic appearance, which consists of an abundant oxyphilic, granular cytoplasm and a large hyperchromatic nucleus, is the result of mitochondrial proliferation that compensates for mitochondrial defects. Mitochondrial function is very important for maintaining functional activity in most endocrine organs; therefore, age-related changes in the mitochondria are likely to impair endocrine organ function. Colloid changes were apparent in 15-month-old and 30-month-old mice. Intrafollicular colloid took on the appearance of a large Tg globule or of numerous, small, fragmented Tg globules within oncocytic follicles. A large globule or multimeric Tg globules are more frequently present in hypofunctioning follicles than in active follicles. In aged mice, we observed oncocytic follicular epithelia lining lumens lacking colloid, which is a characteristic sign of an inactive follicle. We found that follicles composed of oncocytic epithelium are more likely to be irregular in shape, lack colloid, or have highly insoluble colloid. These results demonstrate the important role of mitochondrial function in maintaining proper activity of the thyroid follicular cell.

With advancing age, conversion of functional tissue to fatty or fibrous tissue occurs in most organs, and fundamental tissue loss in a variety of organs is associated with a decrease in proper function. In the aged mouse, however, there is a paucity of conversion to fatty or fibrous tissue. Instead, cystic atrophy is common. Although conversion of thyroid follicles to fatty or fibrous tissue is occasionally observed in elderly women, the proportion of fatty or fibrous tissue within thyroid is not so pronounced.

In conclusion, follicular cells and the follicles of aged thyroids show characteristic morphological changes, which include cystic atrophy, empty colloid, Tg globules, and oncocytic follicular cells.

**Conflicts of Interest**

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

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Comparison of the FDA and ASCO/CAP Criteria for HER2 Immunohistochemistry in Upper Urinary Tract Urothelial Carcinoma

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Background: Human epidermal growth factor receptor 2 (HER2) is one of the known oncogenes in urothelial carcinoma. However, the association between HER2 and the prognosis of upper urinary tract urothelial carcinoma (UUTUC) has not yet been fully clarified. The aim of this study was to evaluate HER2 expression using the United States Food and Drug Administration (FDA) criteria and American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) criteria and compare their prognostic significance in UUTUC. Methods: HER2 expression was evaluated in 144 cases of UUTUC by immunohistochemistry (IHC) using tissue microarrays. We separately analyzed HER2 expression using the FDA and ASCO/CAP criteria. The IHC results were categorized into low (0, 1+) and high (2+, 3+) groups. Results: Using the FDA criteria, 94 cases were negative, 38 cases were 1+, nine cases were 2+, and three cases were 3+. Using the ASCO/CAP criteria, 94 cases were negative, 34 cases were 1+, 13 cases were 2+, and three cases were 3+. Four cases showing 2+ according to the ASCO/CAP criteria were reclassified as 1+ by the FDA criteria. High HER2 expression by both the FDA criteria and ASCO/CAP criteria was significantly associated with International Society of Urological Pathology high grade (p = .001 and p < .001). The high HER2 expression group classified with the FDA criteria showed significantly shorter cancer-specific survival (p = .004), but the HER2 high and low expression groups classified with the ASCO/CAP criteria did not show significant differences (p = .161) in cancer-specific survival. Conclusions: HER2 high expression groups were significantly associated with shorter cancer-specific survival, and our study revealed that the FDA criteria are more suitable for determining HER2 expression in UUTUC.

Key Words: Upper urinary tract urothelial carcinoma; HER2; FDA criteria; ASCO/CAP criteria

Upper urinary tract urothelial carcinoma (UUTUC), urothelial carcinoma of the renal pelvis and ureter, is a relatively rare malignant tumor, and is known to have a less favorable prognosis because of its late diagnosis compared with urothelial carcinoma of the urinary bladder.1,3

Human epidermal growth factor receptor type 2 (HER2) is a transmembrane receptor tyrosine kinase.1 HER2 protein overexpression is well known to be associated with poor prognosis in various cancers, and chemotherapy for HER2 has been established for breast cancers,7,8 gastric cancers, and gastroesophageal junction carcinomas.7,9 In HER2 immunohistochemical staining (IHC), a 3+ HER2 IHC score is regarded as positive, and patients with breast cancer who have a 3+ HER2 IHC score are eligible for trastuzumab therapy, but 2+ HER2 IHC score is regarded as an equivocal result and requires further testing.10,11 Many studies have attempted to clarify more accurate guidelines for interpreting HER2 protein expression in various cancers. In breast cancers, the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) and United States Food and Drug Administration (FDA) guidelines for HER2 testing, including immunohistochemistry, have been studied and used for years. However, these two guidelines have somewhat different criteria for HER2 IHC.12

There have been many reports with respect to HER2 protein overexpression in urothelial carcinomas, and the frequency of HER2 overexpression in urothelial carcinoma is approximately 10%.13,14 Some studies showed that HER2 overexpression was a significant prognostic factor in urothelial carcinoma,15 and recently, HER2 has been regarded as a new therapeutic target for urothelial carcinomas.16 However, a relatively small number of studies have reported HER2 protein expression in UUTUC compared with urothelial carcinoma of the urinary bladder.1,3,16

The prognostic significance of HER2 status in UUTUC is still controversial. A few studies showed that HER2 overexpression
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could predict disease progression and disease-related survival in upper urothelial carcinoma, but another study could not show the prognostic significance of HER2 protein expression. Furthermore, there has been no study comparing the FDA and ASCO/CAP guidelines in HER2 IHC of UUTUC.

Because the association between HER2 and the prognosis of UUTUC has been controversial, we aimed to compare the prognostic significance of HER2 protein expression using the FDA criteria and the ASCO/CAP guidelines to evaluate which one is more valuable to evaluate the prognosis in UUTUC.

MATERIALS AND METHODS

Case selection and tissue microarray construction

A total of 148 cases of UUTUC diagnosed in the Department of Pathology, Seoul National University Hospital, were collected from the computerized database between 1998 and 2005. Hematoxylin and eosin–stained slides were reviewed to determine the pathological diagnosis, tumor grading and pTNM staging compared with the pathology report using the World Health Organization (WHO) classification and American Joint Committee on Cancer (AJCC) staging system. Four cases were excluded because of an inadequate amount of tissue, and eventually, 144 cases were included. The clinical records including age, sex, recurrence, metastasis and survival were collected. We produced tissue microarray (TMA) blocks from formalin-fixed, paraffin-embedded tissue blocks (SuperBioChips Laboratories, Seoul, Korea). Two representative core sections (2 mm in diameter) were taken from the viable tumor area. This study was approved by the Institutional Review Board (IRB) of Seoul National University Hospital.

HER2 immunohistochemistry

HER2 IHC was performed with the HercepTest kit (Dako, Glostrup, Denmark) according to the manufacturer’s protocols. We cut 4-μm-thick sections from the TMA block for HER2 IHC. HER2 expression was separately analyzed using FDA and ASCO/CAP 2013 criteria. Using the FDA criteria, the 2+ positive cases exhibited weak-to-moderate complete membrane staining in >10% of tumor cells, whereas the ASCO/CAP 2013 criteria defined the 2+ positive cases as circumferential membrane staining that is incomplete and/or weak-to-moderate and present within >10% of the invasive tumor cells, or complete and circumferential membrane staining that is intense and present within ≤10% of the tumor cells. The HER2 IHC results were subcategorized into low (0, 1+) and high (2+, 3+) expression groups.

Statistical analysis

Fisher exact test and Pearson’s chi-square test were performed to analyze the correlations between the clinicopathological characteristics and HER2 expression. The cancer-specific survival period was measured from the time of primary surgery to cancer-related death or the last follow-up. The Kaplan-Meier curves were plotted and the log-rank test was used for the univariate analysis of cancer-specific survival. Cox proportional hazards models was used for the multivariate analysis. In all tests, a two-tailed p < .05 was considered statistically significant. SPSS software was used for the statistical analysis (IBM SPSS ver. 21.0, IBM Co., Armonk, NY, USA).

RESULTS

Clinical and pathological characteristics

A total of 144 UUTUC cases were included in the present study, of which 108 cases were male and 36 cases were female. The mean age was 66 years old (range, 43 to 87 years). The average tumor size was 4.2 cm (range, 0.2 to 14.0 cm). Lymph node metastasis was observed in nine cases, and distant metastasis was observed in two cases at the time of surgery. Of the 144 cases, 14 were categorized as pTNM stage 0 (9.7%), 36 as stage 1 (25.0%), 23 as stage 2 (16.0%), 39 as stage 3 (27.1%), and 32 as stage 4 (22.2%). Sixty-one cases were International Society of Urological Pathology (ISUP) low grade and 83 cases were ISUP high grade. The mean follow-up period was 79 months (range, 2 to 201 months).

HER2 immunohistochemistry

Using the FDA criteria, 94 cases (65.3%) were negative, 38 cases (26.4%) had a score of 1+, nine cases (6.3%) were 2+, and three cases (2.1%) were 3+ (Fig. 1). Using the ASCO/CAP criteria, 94 cases (65.3%) were negative, 34 cases (23.6%) were 1+, 13 cases (9.0%) were 2+, and three cases (2.1%) were 3+. Four cases showing 2+ IHC result according to the ASCO/CAP 2013 criteria were reclassified as 1+ by the FDA criteria due to the incomplete membrane staining (Fig. 2).

Correlation between HER2 expression and the clinicopathological characteristics

The relationship between HER2 expression and the clinicopathological parameters is shown in Table 1. ISUP high grade was associated with high HER2 expression using both the FDA criteria (p = .001) and ASCO/CAP 2013 criteria (p < .001). Other clinical and pathologic characteristics such as age (p > .990) and p =
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Fig. 1. Representative images of human epidermal growth factor receptor 2 (HER2) expression using the United States Food and Drug Administration criteria. (A, B) 1+ HER2 immunohistochemistry (IHC) positivity, faint membrane staining in more than 10% of tumor cells. (C, D) 2+ HER2 IHC positivity, moderate complete membrane staining in more than 10% of tumor cells. (E, F) 3+ HER2 IHC positivity, strong, complete membrane staining in more than 10% of tumor cells.

Fig. 2. One of four cases with discrepant scores according to the United States Food and Drug Administration (FDA) and American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) 2013 criteria. This case is classified as 2+ by the ASCO/CAP guidelines, showed moderate and “incomplete” membrane staining in more than 10% of tumor cells, and is classified as 1+ by the FDA guidelines due to the absence of “complete” membrane staining.

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.547), age (p = .761 and p = .430), size (p = .374 and p = .289), T stage (p > .990 and p = .604), N stage (p = .554 and p > .990), M stage (p > .990 and p > .990), and pTNM stage (p = .560 and p > .990) were not associated with HER2 expression using both the FDA and ASCO/CAP 2013 criteria.

Survival analysis

The ISUP high grade, pTNM high stage, and HER2 high expression groups, as classified by the FDA criteria, were significantly associated with a shorter cancer-specific survival (Fig. 3A–C). However, HER2 high expression group, as classified by the ASCO/CAP 2013 criteria, did not show a significant association with a shorter cancer-specific survival (p = .161) (Fig. 3D).

In the multivariate Cox regression analysis, pTNM and HER2 expression, as determined by using the FDA criteria, were independent prognostic factors for cancer-specific survival (Table 2).

DISCUSSION

In this study, we evaluated HER2 protein expression by immunohistochemistry in 144 cases of UUTUC using the following two guidelines: the FDA criteria and ASCO/CAP 2013 criteria. The definitions of IHC 1+ and 2+ are different between the two guidelines, and the IHC results from two guidelines showed dif-
Her2 expression in UUTCC

different prognostic significance in this study. The numbers of 1+ and 2+ positive cases were different, depending on the scoring system. Four cases that scored 2+ using the ASCO/CAP 2013 criteria were reclassified as 1+ by the FDA criteria. This discrepancy resulted from differences in the definitions of 2+ and 1+ positive cases between the two criteria. The FDA criteria classified the 2+ positive cases as having weak-to-moderate “complete” membrane staining in more than 10% of tumor cells, whereas the ASCO/CAP 2013 criteria defined the 2+ positive cases as having circumferential membrane staining that is “incomplete” and/or weak-to-moderate and present within > 10% of the invasive tumor cells, or complete and circumferential membrane staining that is intense and present within ≤ 10% of the invasive tumor cells. In previous studies, the rates of 2+/3+ HER2 IHC in UUTUC ranged from 14% to 18%. In this study, the 2+/3+ HER2 IHC rate was somewhat lower than those of previous studies (8.3% FDA criteria and 11.1% ASCO/CAP 2013 criteria in this study).

The high pTNM stage, high ISUP grade, and high HER2 expression group, as classified by the FDA criteria, showed significantly shorter cancer-specific survival, whereas HER2 expression classified using the ASCO/CAP 2013 criteria did not show a significant association with shorter cancer-specific survival. The multivariate Cox regression analysis indicated that the HER2 IHC results classified by the FDA criteria could be an independent prognostic factor for cancer-specific survival in patients with UUTUC. These results suggest that the FDA guidelines would be more suitable for analyzing HER2 IHC in UUTUC than the ASCO/CAP 2013 guidelines. We think that these results may be important for predicting survival in actual clinical settings.

Some studies have tried to clarify the association between HER2 status and the prognosis of urothelial carcinoma, and the results were diverse. Langner et al. reported that patients with score 2+ with the FDA guidelines had a significant correlation with metastasis-free survival. Vershasselt-Crinquette et al. used the ASCO/CAP criteria and their result showed that HER2 overexpression was not correlated with specific survival or recurrence. Sasaki et al. revealed that HER2 positive patients showed a significant association with shorter time to recurrence using their own criteria according to the ASCO/CAP criteria and the results of the Trastuzumab for Gastric Cancer (ToGA)

| Characteristic | HER2 score | FDA criteria | p-value | ASCO/CAP 2013 criteria | p-value |
|---------------|------------|--------------|---------|------------------------|---------|
|               |            | 0 and 1+     | 2+ and 3+ | 0 and 1+            | 2+ and 3+ | |
| Sex           |            | 0.990        | 0.971    | 0.547                  |          |
| Male          |            | 99           | 9        | 97                     | 11       |
| Female        |            | 33           | 3        | 31                     | 5        |
| Age (yr)      |            | 0.761        |          | 0.430                  |          |
| <66           |            | 56           | 4        | 55                     | 5        |
| ≥66           |            | 76           | 8        | 73                     | 11       |
| Size (cm)     |            | 0.374        |          | 0.289                  |          |
| <4.2          |            | 80           | 9        | 77                     | 12       |
| ≥4.2          |            | 52           | 3        | 51                     | 4        |
| T stage       |            | 0.990        |          | 0.604                  |          |
| Ta, Tis and T1, T2 | | 73 | 7 | 70 | 10 |
| T3 and T4     |            | 59           | 5        | 58                     | 6        |
| N stage       |            | 0.554        |          | 0.990                  |          |
| NO            |            | 124          | 11       | 120                    | 15       |
| N1, N2, and N3|            | 8            | 1        | 8                      | 1        |
| M stage       |            | 0.990        |          | <0.001                 |          |
| MO            |            | 130          | 12       | 126                    | 16       |
| M1            |            | 2            | 0        | 2                      | 0        |
| pTNM stage    |            | 0.990        |          | 0.990                  |          |
| OA, OIs, I and II |       | 68 | 5 | 65 | 8 |
| III and IV    |            | 64           | 7        | 63                     | 8        |
| ISUP          |            | 0.001        |          | <0.001                 |          |
| Low           |            | 61           | 0        | 61                     | 0        |
| High          |            | 71           | 12       | 67                     | 16       |

HER2, human epidermal growth factor receptor 2; FDA, United States Food and Drug Administration; ASCO/CAP, American Society of Clinical Oncology/College of American Pathologists; ISUP, International Society of Urological Pathology.
trial. Tsai et al.\textsuperscript{3} also used their own scoring system and showed the result that HER2 expression predicted for disease progression and disease-related survival. We thought that the diverse results could be caused by the different scoring system for HER2 IHC. Furthermore, there has been no study that compared the FDA and ASCO/CAP guidelines for interpretation of HER2 IHC in

|   | Hazard ratio (95% CI) | p-value |
|---|----------------------|---------|
| ISUP grade | High vs Low | 1.710 (0.739–3.958) | .210 |
| pTNM stage | III, IV vs 0, I, II | 5.153 (2.267–11.715) | <.001 |
| HER2 expression (FDA) | High vs Low | 2.478 (1.073–5.724) | .034 |

CI, confidence interval; ISUP, International Society of Urological Pathology; HER2, human epidermal growth factor receptor 2; FDA, United States Food and Drug Administration.

Fig. 3. Kaplan-Meier survival curves for cancer-specific survival. (A) Cancer-specific survival according to the International Society of Urological Pathology (ISUP) grade. (B) Cancer-specific survival according to the pTNM stage. (C) Cancer-specific survival according to human epidermal growth factor receptor 2 (HER2) expression by the United States Food and Drug Administration (FDA) criteria. (D) Cancer-specific survival according to HER2 expression by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) criteria.
UUTUC. In this study, we compared the FDA and ASCO/CAP guidelines for HER2 IHC in UUTUC, and found that the prognostic significance of HER2 IHC was different between the two guidelines.

We did not confirm the HER2 gene amplification status by fluorescence in situ hybridization analysis, and this is the limitation of the present study. There have been some studies reporting good correlation between HER2 protein overexpression and gene amplification in UUTUC.\textsuperscript{2,13}

In conclusion, our study indicated that the HER2 IHC result has significant prognostic implications and the FDA criteria are more suitable for interpreting the HER2 IHC results in UUTUC than the ASCO/CAP criteria in UUTUC.

**Conflicts of Interest**

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

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Detection of Human Papillomavirus in Korean Breast Cancer Patients by Real-Time Polymerase Chain Reaction and Meta-Analysis of Human Papillomavirus and Breast Cancer

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Background: Human papillomavirus (HPV) is a well-established oncogenic virus of cervical, anogenital, and oropharyngeal cancer. Various subtypes of HPV have been detected in 0% to 60% of breast cancers. The roles of HPV in the carcinogenesis of breast cancer remain controversial. This study was performed to determine the prevalence of HPV-positive breast cancer in Korean patients and to evaluate the possibility of carcinogenic effect of HPV on breast. Methods: Meta-analysis was performed in 22 case-control studies for HPV infection in breast cancer. A total of 123 breast cancers, nine intraductal papillomas and 13 nipple tissues of patients with proven cervical HPV infection were tested by real-time polymerase chain reaction to detect 28 subtypes of HPV. Breast cancers were composed of 106 formalin-fixed and paraffin embedded (FFPE) breast cancer samples and 17 touch imprint cytology samples of breast cancers. Results: The overall odds ratio between breast cancer and HPV infection was 5.43 (95% confidence interval, 3.24 to 9.12) with I² = 34.5% in meta-analysis of published studies with case-control setting and it was statistically significant. HPV was detected in 22 cases of breast cancers (17.9%) and two cases of intraductal papillomas (22.2%). However, these cases had weak positivity. Conclusions: These results failed to serve as significant evidence to support the relationship between HPV and breast cancer. Further study with larger epidemiologic population is merited to determine the relationship between HPV and breast cancer.

Key Words: Breast neoplasms; Human papillomavirus; Real-time polymerase chain reaction; Meta-analysis
MATERIALS AND METHODS

Meta-analysis

A systematic literature search was conducted in PubMed (January 1, 1992, to September 30, 2015) with the following keywords: “breast neoplasm” and “human papillomavirus.” All potentially relevant studies were reviewed. Studies with case-control setting were selected. Analyses were performed using R ver. 3.2.2 (2015-08-14) statistical software.

Tumor samples

The study was performed with 123 breast cancers and nine intraductal papillomas. These patients received surgeries at Korea University Guro Hospital from January 2007 to January 2015. The study protocol was approved by the Institutional Review Board of Guro Hospital. The 123 breast cancer samples were composed of 106 formalin-fixed and paraffin embedded (FFPE) tissues and 17 touch imprint cytology samples. The patients who received mastectomy and nipple resection were preferentially selected to obtain FFPE nipple tissues. The touch imprint cytology samples were also used to compare FFPE tissues and cytology samples. The nine intraductal papillomas samples were composed of nine FFPE tissues. To define the infection route by detecting HPV in nipple, 13 FFPE nipple tissues of breast cancer patients who had been confirmed with HPV infection in uterine cervix were included in this study. All materials were obtained from the tissue bank of Korea University Guro Hospital. Medical records and pathological reports of patients and histological features of breast cancer enrolled in this study were reviewed.

DNA isolation

Two or three 10-μm sections were taken from FFPE tissues. Deparaffinization of these sections was sufficiently done by xylene and ethanol treatment for 5 minutes alternately for three times. DNA was extracted using QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instruction. The concentration of extracted DNA was measured on Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). The quality of DNA was determined by evaluating A260/A280 ratio. DNA isolation from touch imprint sample from fresh breast cancer was also performed with QIAamp DNA Mini Kit according to the manufacturer’s instruction.

Table 1. The published studies of HPV and breast cancer in case-control setting

| Study          | Country       | Case (n=1,833) | Control (n=893) |
|----------------|---------------|---------------|----------------|
|                |               | HPV (+)       | HPV (-)        | HPV (+)         | HPV (-)        |
| Bratthauer et al. (1992) | USA           | 0             | 28             | 0              | 15             |
| Yu et al. (1999)     | China, Japan  | 18            | 34             | 1              | 19             |
| Damin et al. (2004)  | Brazil        | 25            | 76             | 0              | 41             |
| Tsai et al. (2005)   | Taiwan        | 8             | 54             | 2              | 42             |
| Choi et al. (2007)   | Korea         | 8             | 115            | 0              | 31             |
| de Leon et al. (2009)| Mexico        | 15            | 36             | 0              | 43             |
| Ho et al. (2009)     | China         | 24            | 16             | 1              | 19             |
| Hsing et al. (2009)  | Australia     | 8             | 20             | 3              | 25             |
| Mendizabal-Ruiz et al. (2009) | Mexico   | 3             | 64             | 0              | 40             |
| Mou et al. (2011)    | China         | 4             | 58             | 0              | 46             |
| Chang et al. (2012)  | China         | 0             | 48             | 0              | 30             |
| Freig et al. (2012)  | Italy         | 9             | 22             | 0              | 12             |
| Glenn et al. (2012)  | Australia     | 35            | 42             | 11             | 47             |
| Siganoodi et al. (2012) | Iran        | 15            | 43             | 1              | 40             |
| Liang et al. (2013)  | China         | 48            | 176            | 6              | 31             |
| All et al. (2014)    | Iraq          | 60            | 69             | 3              | 41             |
| Ahangar-Oskoue et al. (2014) | Iran | 22           | 43             | 0              | 65             |
| Manzouri et al. (2014)| Iran        | 10            | 45             | 7              | 44             |
| Peng et al. (2014)   | China         | 2             | 98             | 0              | 50             |
| Fu et al. (2015)     | China         | 25            | 144            | 1              | 82             |
| Vernet-Tomas et al. (2015) | Spain   | 0             | 76             | 0              | 2              |
| Li et al. (2015)     | China         | 3             | 184            | 0              | 92             |
| Total               |               | 342           | 1491           | 36             | 857            |

HPV, human papillomavirus.
Real-time polymerase chain reaction

Extracted DNA samples were subject to real-time polymerase chain reaction (PCR) with gene specific primers provided with Anyplex II HPV 28 Detection System (Seegene, Seoul, Korea) using CFX96 Real-Time PCR (Bio-Rad, Hercules, CA, USA). Melting curves were analyzed using the exclusive analysis program provided with the Anyplex II HPV 28 Detection System. This system is able to detect 28 subtypes of HPV, including all subtypes reported in the literatures, such as HPV-11, HPV-16, HPV-18, HPV-33, HPV-58, HPV-59, HPV-73, and HPV-82. Because the Anyplex II HPV 28 Detection System was originally designed for cytological samples swabbed in uterine cervix, verification of Anyplex II HPV 28 Detection System with FFPE tissue of uterine cervical squamous cell carcinoma was performed.

RESULTS

Meta-analysis

Twenty-two case-control studies for HPV infection in breast cancer were enrolled in meta-analysis with random effect model. HPV infection was detected in 342 of 1,833 breast cancers and in 36 of 857 benign breast lesions (Table 1). The overall odds ratio between breast cancer and HPV infection was 5.43 (95% confidence interval, 3.24 to 9.12) with F = 54.5% (Fig. 1). This result was statistically significant.

Clinicopathologic data

The median age of 123 breast cancer patients was 51.6 years (range, 23 to 79 years). The 123 breast cancers included 103 invasive carcinomas of no special type, five invasive lobular carcinomas, one microinvasive carcinoma, three ductal carcinomas in situ, and 11 carcinomas of other specific subtypes. Other clinicopathological characteristics of the 123 cases of breast cancer are summarized in Table 2.

Of the 13 cases whose FFPE nipple tissues were tested for HPV, one case was positive for HPV-16 infection in uterine cervix and 12 cases were positive for HPV infection in uterine cervix by Hybrid Capture 2 (Qiagen, Gaithersburg, MD, USA) (Table 3). Of these 13 cases, five had low-grade squamous intraepithelial lesions, one had high-grade squamous intraepithelial lesion, and one had endocervical type adenocarcinoma in uterine cervix.

![Fig. 1. The forest plot for relationship between breast cancer and human papillomavirus infection in case-control setting. OR, odds ratio; CI, confidence interval.](https://doi.org/10.4132/jptm.2016.07.08)
Availability of Anyplex II HPV 28 Detection System in FFPE tissue

HPV-16 was detected in the FFPE tissue of uterine cervical squamous cell carcinoma using Anyplex II HPV 28 Detection System, in concordance with the result of cytology sample using Anyplex II HPV 28 Detection System, proving that Anyplex II HPV 28 Detection System worked properly for FFPE tissue as for the cytology specimen. Therefore, Anyplex II HPV 28 Detection System can be used to detect HPV infection in FFPE tissue.

Real-time PCR

Proper DNA samples were isolated from 106 FFPE breast cancers, 17 touch imprint cytology samples of breast cancer, 13 FFPE nipple tissues, and nine FFPE intraductal papillomas. Amplification of dissociation curve of HPV subtypes 6, 16, 33, 39, 40, 51, 53, 58, and 61 was detected in real-time PCR of 22 FFPE breast cancers (17.9%) (Fig. 2). The 22 cases included 19 cases of invasive carcinoma of no special type, one case of adenoid cystic carcinoma, one case of metaplastic carcinoma, and one case of apocrine carcinoma (Table 4). Histological features of HPV infection were not identified by slide review of these 22 cases. In two FFPE tissues of intraductal papilloma, amplification of dissociation curve of HPV-33 and HPV-53 was detected in real-time PCR (22.2% of nine intraductal papilloma FFPE tissues).

However, because HPV-positive cases showed weak amplification of the dissociation curve, these results were considered as weak positivity for HPV. HPV was not detected in 17 touch imprint cytology samples of breast cancer or 13 FFPE tissues of nipple.

DISCUSSION

Genetic and environmental factors such as mutation in BRCA1/2, ethnicity, hormonal effect, diet, and ionizing radiation are known to be involved in the carcinogenesis of breast cancer.

Table 2. Clinicopathological characteristics of 123 cases of breast cancer

| Characteristic       | Criteria             | No. |
|----------------------|----------------------|-----|
| Age                  | 34–50                | 67  |
|                      | 51–66                | 56  |
|                      | Median (range)       | 51.6 (23–79) |
| Operation type       | Mastsectomy          | 106 |
| Histologic grade     | Grade 1              | 27  |
|                      | Grade 2              | 41  |
|                      | Grade 3              | 52  |
| Hormonal receptor    | ER positive          | 69  |
|                      | PR positive          | 62  |
|                      | HER2 positive        | 52  |
|                      | Triple negative      | 25  |
| Lymph node metastasis| Positive             | 54  |
| HPV detection in cervix| Positive            | 54  |
|                      | Negative             | 69  |
| Histologic type      | Invasive carcinoma of no special type | 103 |
|                      | Invasive lobular carcinoma | 5  |
|                      | Micronvasive carcinoma | 1  |
|                      | Ductal carcinoma in situ | 3  |
|                      | Metaplastic carcinoma | 2  |
|                      | Carcinoma with medullary feature | 2  |
|                      | Apocrine carcinoma   | 2   |
|                      | Carcinoma with neuroendocrine feature | 1  |
|                      | Adenoid cystic carcinoma | 1  |
|                      | Mucinous carcinoma   | 1   |
|                      | Micropapillary carcinoma | 1  |

Table 3. Clinicopathologic data of breast cancer patients confirmed HPV infection in uterine cervix

| Case No. | Age of breast surgery (yr) | Histology of breast | Age of HPV detection at uterine cervix (yr) | Histology of uterine cervix | Method          |
|----------|---------------------------|---------------------|---------------------------------------------|-----------------------------|-----------------|
| 15       | 50                        | Invasive carcinoma of NST | 54                          | NI                          | HC2             |
| 16       | 44                        | Metaplastic carcinoma  | 44                           | LSIIL                       | HC2             |
| 17       | 43                        | Invasive carcinoma of NST | 44                          | LSIIL                       | HC2             |
| 18       | 48                        | Invasive carcinoma of NST | 48                          | NI                          | HC2             |
| 19       | 50                        | Invasive carcinoma of NST | 50                          | NI                          | HC2             |
| 20       | 46                        | Invasive carcinoma of NST | 46                          | LSIIL                       | HC2             |
| 21       | 43                        | Invasive carcinoma of NST | 41                          | NI                          | HC2             |
| 22       | 49                        | Invasive carcinoma of NST | 49                          | NI                          | HC2             |
| 23       | 44                        | Invasive carcinoma of NST | 44                          | LSIIL                       | HC2             |
| 24       | 63                        | Invasive carcinoma of NST | 60                          | Adenocarcinoma, endocervical type | HC2             |
| 25       | 50                        | Invasive carcinoma of NST | 50                          | NI                          | Medical record (HPV-16) |
| 26       | 56                        | Invasive carcinoma of NST | 47                          | LSIIL                       | HC2             |
| 27       | 57                        | Invasive carcinoma of NST | 46                          | HSIL                        | HC2             |

HPV, human papillomavirus; NST, no special type; NI, in the biopsy, uterine cervical lesion was not identified; HC2, Hybrid Capture 2 (QIAGEN, Gaithersburg, MD, USA) in cytology sample; LSIIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions.
However, two-thirds of patients with breast cancer have no association with these risk factors. To elucidate viral carcinogenesis in mammary cancer development, many studies have focused on oncogenic virus. According to multistep carcinogenesis model of breast cancer, TP53 is considered as one predisposing gene. Because HPV E6 and E7 oncoproteins are able to inactivate TP53 gene,

Fig. 2. The representative results of dissociation curve in real-time polymerase chain reaction. (A) Weak positivity for human papillomavirus (HPV) 33. The dissociation curve of two cases shows the low melting peak. (B) Negative case. The dissociation curve of almost cases do not show the melting peaks. (C) Positive control. The dissociation curve of positive control shows 28 melting peaks about 28 subtypes of HPV.
HPV can play a role in this multistep mammary carcinogenesis. On the other hand, Ohba et al.\(^\text{38}\) have suggested that HPV infection induces overexpression of APOBEC3B associated with the early stage of carcinogenesis in breast cancer.

Recently, many studies have been performed to prove the relationship between breast cancer and HPV infection. These studies have revealed that approximately 19.8% of HPV infections are in breast cancer (range, 0% to 60%). The causes of no detection of HPV infection include geographic factors due to race and prevalence of HPV infection, selection bias due to difference in prevalence, and too low viral load to be technically detectable. At present, most studies performed in China and Middle East have showed high prevalence of positivity for HPV in breast cancer.

Meta-analysis from case-control setting revealed that the prevalence of HPV infection in breast cancer was higher than that in benign breast lesion. The \( I^2 \) was appropriate for random effect model. The overall odds ratio between breast cancer and HPV infection was statistically significant. However, there are some limitations of meta-analysis. First, publication bias might

### Table 4. Clinicopathologic data and HPV subtype of HPV-positive breast cancer

| Case No. | Age (yr) | Sample | Histologic type | HPV subtype |
|----------|----------|--------|-----------------|-------------|
| 1        | 50       | FFPE   | Invasive carcinoma of NST | 33          |
| 3        | 46       | FFPE   | Invasive carcinoma of NST | 33          |
| 8        | 55       | FFPE   | Adenoid cystic carcinoma | 40, 51, 61  |
| 9        | 63       | FFPE   | Metaplastic carcinoma    | 40, 51, 53, 61 |
| 17       | 23       | FFPE   | Invasive carcinoma of NST | 40, 51, 53, 61 |
| 20       | 32       | FFPE   | Invasive carcinoma of NST | 51, 53     |
| 30       | 47       | FFPE   | Invasive carcinoma of NST | 40, 51, 58 |
| 39       | 44       | FFPE   | Invasive carcinoma of NST | 51          |
| 40       | 40       | FFPE   | Invasive carcinoma of NST | 33, 51, 53 |
| 41       | 49       | FFPE   | Invasive carcinoma of NST | 51, 53     |
| 49       | 55       | FFPE   | Invasive carcinoma of NST | 51          |
| 50       | 38       | FFPE   | Invasive carcinoma of NST | 51          |
| 63       | 70       | FFPE   | Invasive carcinoma of NST | 6, 51, 58  |
| 73       | 42       | FFPE   | Invasive carcinoma of NST | 6, 40, 53  |
| 74       | 58       | FFPE   | Invasive carcinoma of NST | 6, 51     |
| 75       | 54       | FFPE   | Invasive carcinoma of NST | 51          |
| 81       | 65       | FFPE   | Invasive carcinoma of NST | 6, 40     |
| 93       | 35       | FFPE   | Invasive carcinoma of NST | 53          |
| 99       | 54       | FFPE   | Invasive carcinoma of NST | 6          |
| 109      | 50       | FFPE   | Invasive carcinoma of NST | 51          |
| 120      | 63       | FFPE   | Apocrine carcinoma      | 16          |
| 122      | 50       | FFPE   | Invasive carcinoma of NST | 39          |

HPV, human papillomavirus; FFPE, formalin-fixed and paraffin embedded tissue; NST, no special type.
exist due to low prevalence of HPV infection in breast cancers. Many studies that failed to detect HPV in breast cancer might not have been reported in the literature. Moreover, because 22 studies were performed with different methods that could only detect limited HPV subtypes, the prevalence of HPV infection in breast cancer might have been underestimated. Therefore, the quality of data from the literature might be questionable.

We found that variable HPV subtypes were detected in 22 of 123 Korean breast cancers by real-time PCR. Specific HPV subtypes including HPV-39, HPV-40, HPV-53, and HPV-61 have not been mentioned in the literature. HPV-51 was the most frequently found subtype. It was detected in 14 of 22 HPV-positive breast cancers. HPV-16 and HPV-18 were the most frequently found subtypes in cervical cancer and oropharyngeal cancer. However, they were not common HPV subtypes in breast cancer. They were not detected in the two intraductal papillomas either. If HPV infection is involved in carcinogenesis of breast, specific HPV subtypes hardly related in cervical cancer and oropharyngeal cancer can have a role as carcinogen in breast. Interestingly, this result showed that the prevalence of HPV infection in benign breast lesions (22.2%) was higher than that in breast cancers (17.9%), although meta-analysis supported correlation between breast cancer and HPV. However, because only nine intraductal papillomas were tested, this result might have been compromised.

It is important to note that most cases showed weak positivity for HPV. Fragmentation of extracted DNA in FFPE tissues could be one of the reasons responsible for this result. However, even when the reduction of positivity by fragmentation was considered, positivity for HPV in FFPE breast cancer samples was too weak. Khan et al. have also found HPV DNA in 26 of 124 Japanese breast cancer patients by PCR for FFPE breast cancer samples. Because the viral load in breast cancers was very low compared to viral load examined in uterine cervical carcinoma, they concluded that HPV was not involved in the development of breast cancers in Japanese. Due to the weak positivity of HPV in this study, we also concluded that this result was not a significant evidence to support carcinogenesis of HPV in breast.

If HPV is involved in the development of breast cancer, some differences between HPV-positive and HPV-negative breast cancers should be present. Heng et al. have found putative koilocytes as the proof of HPV infection in HPV-positive breast cancers. However, Khan et al. reviewed all HPV-positive breast cancer and failed to find koilocytes. In this study, we also failed to find koilocytes after slide review of the 123 breast cancers and nine intraductal papillomas. Kan et al. have reported that HPV-positive breast cancers are not correlated with grade, patient survival, hormonal receptor status, HER-2 expression, or p53 overexpression. The characteristics of HPV-positive breast cancer are still controversial.

Since HPV is known as a sexually transmitted virus, HPV may be transmitted to the breast tissue through nipple by sexual behavior. We didn’t detect HPV infection in 13 nipple FFPE tissues and FFPE breast cancers of patients who had HPV infection in uterine cervix (Table 3) and failed prove that nipple was the infection route. However, Glenn et al. have suggested that HPV can be detected in the epithelial cells extracted from human milk. In addition, de Villiers et al. have detected HPV infection in nipple tissues. Some studies have tried to prove the coexistence of HPV infection in both cervical lesion and breast cancer. Hennig et al. have proved that HPV-16 positive breast cancer is corresponding to HPV-16 positive high-grade cervical intraepithelial neoplasia in 19 of 41 cases. On the contrary, Lv et al. failed to find coexistence of HPV in breast or cervical tissues of 12 cases. In our study, there was no case of HPV coexistence in cervical lesion or breast cancer. Furthermore, although most cervical and oropharyngeal cancers are provoked by HPV-16 and HPV-18, and HPV-16 was found in one breast cancer only. Common infected HPV subtypes of breast cancer might be different from those of cervical and oropharyngeal cancers. It is not convincing that the cervical intraepithelial neoplasia is one of the risk factors of breast cancer.

Statistical significance of correlation between breast cancer and HPV was found in meta-analysis using published studies of case-control setting. In addition, we detected 22 HPV-positive breast cancers in 123 Korean patients and two HPV-positive intraductal papillomas in nine Korean patients without finding histological characteristics of HPV infection in breast cancers. Because all HPV-positive breast cancers showed weak positivity, a correlation between HPV and breast cancer was not confirmed in this study. Further study with larger epidemiologic population is necessary to elucidate the role of HPV in mammatory carcinogenesis.

Conflicts of Interest

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

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Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis (MTB) that remains one of the world’s most important health problems due to its high risk of complications, mortality, and infectivity. TB was initially considered untreatable, but effective drug regimens that have been developed since the 1940s have increased the treatment success rates to 85%. A rapid and reliable diagnosis of TB is essential to provide proper treatment, avoid severe complications, and prevent disease dissemination.

Among the many traditional diagnostic methods, mycobacterial culture is the gold standard test for diagnosing tuberculosis (TB), but it is time-consuming. Polymerase chain reaction (PCR) is a highly sensitive and specific method that can reduce the time required for diagnosis. The diagnostic efficacy of PCR differs, so this study determined the actual sensitivity of TB-PCR in tissue specimens. Methods: We retrospectively reviewed 574 cases. The results of the nested PCR of the IS6110 gene, mycobacterial culture, TB-specific antigen-induced interferon-γ release assay (IGRA), acid-fast bacilli (AFB) staining, and histological findings were evaluated. Results: The positivity rates were 17.6% for PCR, 3.3% for the AFB stain, 22.2% for mycobacterial culture, and 55.4% for IGRA. PCR had a low sensitivity (51.1%) and a high specificity (86.3%) based on the culture results of other studies. The sensitivity was higher (65.5%) in cases with necrotizing granuloma but showed the highest sensitivity (66.7%) in those with necrosis only. The concordance rate between the methods indicated that PCR was the best method compared to mycobacterial culture, and the concordance rate increased for the methods using positive result for PCR or histologic features. Conclusions: PCR of tissue specimens is a good alternative to detect tuberculosis, but it may not be as sensitive as previously suggested. Its reliability may also be influenced by some histological features. Our data showed a higher sensitivity when specimens contained necrosis, which indicated that only specimens with necrosis should be used for PCR to detect tuberculosis.

Key Words: Tuberculosis; Polymerase chain reaction; Mycobacterial culture; Interferon-γ release tests

Background: Mycobacterial culture is the gold standard test for diagnosing tuberculosis (TB), but it is time-consuming. Polymerase chain reaction (PCR) is a highly sensitive and specific method that can reduce the time required for diagnosis. The diagnostic efficacy of PCR differs, so this study determined the actual sensitivity of TB-PCR in tissue specimens. Methods: We retrospectively reviewed 574 cases. The results of the nested PCR of the IS6110 gene, mycobacterial culture, TB-specific antigen-induced interferon-γ release assay (IGRA), acid-fast bacilli (AFB) staining, and histological findings were evaluated. Results: The positivity rates were 17.6% for PCR, 3.3% for the AFB stain, 22.2% for mycobacterial culture, and 55.4% for IGRA. PCR had a low sensitivity (51.1%) and a high specificity (86.3%) based on the culture results of other studies. The sensitivity was higher (65.5%) in cases with necrotizing granuloma but showed the highest sensitivity (66.7%) in those with necrosis only. The concordance rate between the methods indicated that PCR was the best method compared to mycobacterial culture, and the concordance rate increased for the methods using positive result for PCR or histologic features. Conclusions: PCR of tissue specimens is a good alternative to detect tuberculosis, but it may not be as sensitive as previously suggested. Its reliability may also be influenced by some histological features. Our data showed a higher sensitivity when specimens contained necrosis, which indicated that only specimens with necrosis should be used for PCR to detect tuberculosis.

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so it has become an important diagnostic tool for the identification of MTB. Therefore, nested PCR for IS6110 is now the most effective method to detect TB in formalin-fixed, paraffin-embedded tissue with a high sensitivity (58%–100%) and specificity (75%–100%), but some studies have reported variable PCR sensitivities. As a result, our study was planned to determine the clinical usefulness of nested PCR in practically selected cases and also to compare these results to other methods, including culture results.

MATERIALS AND METHODS

Cases

We retrospectively searched cases in which tissue was obtained. We included patients with a clinical suspicion of TB as well as those without. We selected cases that had been investigated for TB using mycobacterial culture, the TB specific antigen-induced interferon-γ release assay (IGRA), and AFB staining and collected their results to compare with the PCR results of the tissue specimens. In total, 574 formalin-fixed paraffin-embedded samples were obtained from Korea University Anam Hospital (Seoul, Korea) between January 2009 and December 2013. This study was approved by the Institutional Review Board of Korea University Anam Hospital (AN15200-002).

All tissues were fixed in 10% formalin or 4% formaldehyde. We reviewed the slides for their histological findings after hematoxylin and eosin staining. Additional AFB staining was performed on some slides to detect Mycobacterium bacilli. We considered the AFB staining results positive when at least one bacillus was found.

Nested PCR

Tissue preprocessing

Ten pieces of 5–7-μm-thick sections were cut from the formalin-fixed, paraffin-embedded block and collected in 1.5-mL microcentrifuge tubes for the nested PCR. Tissue microdissection was not performed. A 1,000 μL aliquot of 1× phosphate buffered saline was added to remove the paraffin; the mixture was vortex mixed for 10 seconds and then allowed to stand at 75°C for 2 minutes before being centrifuged for 2 minutes at 13,000 rpm. The paraffin and supernatant were removed. These steps were repeated twice more after adding 1 mL of sterile distilled water.

DNA extraction

The DNA was extracted and nested PCR was performed using an NTM&MTB PCR Kit (BioSewoom Inc., Seoul, Korea) according to the manufacturer’s instructions. A 50-μL aliquot of a DNA extraction buffer was added to the preprocessed specimen, and then the mixture was vortex mixed and allowed to stand at 56°C for 15 minutes, followed by thorough mixing. Next, the mixture was placed on a heating block at 100°C for 8 minutes and cooled by mixing for 20 seconds. The mixture was centrifuged for 2 minutes at 13,000 rpm, and the 4-μL upper portion of the supernatant was used.

Nested PCR for IS6110

A 0.5-μL aliquot of enzymes and a mixture of Taq polymerase and buffer were added to 15.5 μL of a mixture of the primers of both MTB and NTM, dNTPs, buffer, and pink dye. Next, 4.0 μL of the extracted DNA were added to the reaction mixture for the first PCR round. Positive and negative controls were used. After the first PCR round, 18.0 μL of the first PCR round product and 0.5 μL of the second PCR round enzyme were mixed with 1.5 μL of the first PCR round product. Both PCR cycles were conducted with an initial 4-minute denaturation step at 95°C, three repeating cycles of 30 seconds each at 94°C, 65°C, and 72°C, followed by 35 cycles of 30 seconds at 94°C, 68°C, and 72°C, and 5 minutes of the final extension at 72°C for 30 seconds. The third cycle was repeated 25 times instead of the 35 cycles that took place during the second PCR round.

PCR products

A 5–10-μL aliquot of the PCR product was resolved on 1.5%–2% agarose gels. The products were visualized by ultraviolet transillumination and compared to the positive control.

Other methods used to detect TB

Different fluid specimens were obtained, decontaminated with 4% NaOH, and used for mycobacterial culture. Whole blood that had been obtained from a patient was used for the IGRA, and the interferon-gamma level was assessed via an enzyme immunoassay.

Statistical analysis

We considered patients with culture-positive results to be patients with TB. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for PCR as well as the other methods. We compared the results from the surgical specimens with those of the biopsy specimens to detect the influence of sampling. Concordance between the results from different methods was assessed using the κ coefficient.
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RESULTS

Clinicopathological characteristics of the cases

The median patient age was 50.3 years (range, 2 to 89 years); 293 cases (51.0%) were male, and 281 participants (49.0%) were female. A total of 239 specimens (41.6%), were obtained by surgical resection, while 335 (58.4%) were procured through biopsy, including 198 (34.5%) from the gastrointestinal tract (colon, stomach, terminal ileum, and duodenum), 108 (18.8%) from the bone, joints, or soft tissue, 86 (15.0%) from the lymph nodes, 73 (12.7%) from the lung or bronchus, 29 (5.1%) from the pleura, and 31 (5.4%) from the skin (Table 1).

Analysis of PCR and other methods compared to culture

PCR was performed on tissue specimens from all cases (n = 574), and 17.6% (n = 101) were positive for TB, whereas 82.4% (n = 473) were negative (Fig. 1C). Among the 574 tissue specimens, 233 underwent AFB staining; only 3.3% (n = 8) had a positive result. In all, 405 cases were cultured, with a positivity rate of 22.2%. The IGRA-positive rate was 55.4% (Table 2).

As mycobacterial culture is the gold standard for TB diagnosis, we calculated the accuracy of each method compared to the mycobacterial culture results to determine the diagnostic yield (Table 3). The PCR sensitivity and specificity were 51.1% and 86.3%, respectively. The PPV was 51.7%, and the NPV was 86.1%, indicating low sensitivity and high specificity compared to prior data.14-20 The sensitivity of AFB-stained specimens was 12.5%, and the specificity was 98.4%. The PPV was 75.0%, while the NPV was 72.4%.

A comparison of PCR and AFB staining of tissue specimens according to their histological features

PCR and AFB staining were performed on the same tissue specimens, so we compared the results of the two methods. Among the eight AFB stain–positive cases (Fig. 1B), six (75.0%) were PCR-positive and two (25.0%) were PCR-negative. Out of the 225 AFB stain–negative cases, 31 (13.8%) were PCR-positive while 194 (86.2%) were PCR-negative.

A review of the hematoxylin and eosin slides (Fig. 1A) revealed the histological findings of necrotizing granulomatous inflammation, which suggests the possibility of TB, in 158 cases (27.5%). Among these, 95 cases (16.6%) had non-necrotizing granulomas, while 45 (7.8%) were diagnosed with necrosis without granuloma. In total, 276 cases (48.1%) had no granuloma or necrosis (Table 1). Based on the culture results, our data showed different diagnostic values depending on the histologic findings.

Specimens with necrotizing granulomatous inflammation had a sensitivity of 65.5%, a specificity of 57.9%, a PPV of 52.9%, and an NPV of 69.8%. For specimens with non-necrotizing granuloma only, the sensitivity was 26.3%, the specificity was 81.0, the PPV was 38.5%, and the NPV was 70.8%, while those specimens with necrosis only had a sensitivity of 66.7%, a specificity of 91.4%, a PPV of 40.0%, and an NPV of 97.0%. The sensitivity was 23.1%, the specificity was 98.8%, the PPV was 60.0%, and the NPV was 94.2% for specimens without granuloma or necrosis. Specimens with necrotizing granulomatous inflammation and with necrosis only showed a higher sensitivity than all others. The latter revealed a higher specificity, while the former had the lowest specificity. The highest specificity was found in specimens without granuloma or necrosis; these cases demonstrated not only the highest PPV but also a high NPV. Specimens with necrotizing granulomatous inflammation were found to have a high PPV, and NPV was also high in specimens with necrosis only.

Table 1. Clinicopathological characteristics of the patients

| Clinicopathological characteristic | No. (%) |
|-----------------------------------|---------|
| Sex                               |         |
| Male                              | 293 (51.0) |
| Female                            | 281 (49.0) |
| The way obtaining tissue          |         |
| Surgical resection                | 239 (41.6) |
| Biopsy                            | 335 (58.4) |
| Histology                         |         |
| Necrotizing granulomatous inflammmation | 158 (27.5) |
| Granulomatous inflammation without necrosis | 95 (16.6) |
| Necrosis without granuloma        | 45 (7.8) |
| No granuloma or necrosis          | 276 (48.1) |
| Site                              |         |
| Gastrointestinal tract            | 198 (34.5) |
| Bone and soft tissue              | 108 (18.8) |
| Lymph node                        | 86 (15.0) |
| Lung and pleura                   | 102 (17.8) |
| Skin                              | 31 (5.4) |
| Peritoneum                        | 18 (3.1) |
| Head and neck                     | 14 (2.4) |
| Brain                             | 3 (0.5) |
| Adnexa, breast, pericardium, spleen, testis | Each 2 (0.3) |
| Liver, kidney, bladder, thymus    | Each 1 (0.17) |
Concordance rate between methods

The concordance rate between the methods was assessed using the \( \kappa \) coefficient. None of the methods had excellent agreement with the cultures. When correlating was carried out based on the culture results, PCR was the best method (\( \kappa = 0.376 \)), and the concordance rate increased for the method that used a positive result for the PCR or histologic features (\( \kappa = 0.388 \)). However, PCR alone had a better concordance rate (\( \kappa = 0.442 \)) with the histological findings (Table 5).

DISCUSSION

TB remains an important health problem, even though the cure rate has increased and the incidence has dropped.6 As a rapid and sensitive method to detect TB, PCR was able to eliminate the disadvantages of mycobacterial culture.3–5 However, efficacy assessments for diagnosing TB by PCR differ. Our study evaluated the use of PCR in paraffin-embedded tissue specimens to diagnose TB.

The sensitivity of PCR for the IS6110 insertion sequence was 51.1% in tissue specimens, which is lower than that reported by previous studies (58%–100%) (Table 6)14–20 and suggests that PCR alone may have had a relatively lower sensitivity for the patients in our study than any previous results in TB-suspected patients.

The low sensitivity of PCR in our study can be explained in several ways. First, the quantity of DNA in the tissue specimens may have been insufficient for a proper evaluation. If a specimen contained a smaller number of bacteria, the bacilli may not have

**Table 2. Positive and negative rates for each method**

| Method                  | Positive | Negative | Total |
|-------------------------|----------|----------|-------|
| PCR                     | 101 (17.6)| 473 (82.4)| 574 (100) |
| AFB stain               | 8 (3.3)  | 225 (96.7) | 233 (100) |
| IGRA                    | 77 (55.4) | 62 (44.6)  | 139 (100) |
| Mycobacterial culture   | 90 (22.2) | 315 (77.8) | 405 (100) |

Values are presented as number (%). PCR, polymerase chain reaction; AFB, acid-fast bacilli; IGRA, tuberculosis-specific antigen-induced interferon gamma release assay.
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been included in the section used for PCR. The possibility of bacilli being contained in a biopsy specimen was lower because of the small sample size. In our study, isolating TB using the PCR resulted in a significantly different sensitivity between the surgical and biopsy specimens (59.5% vs 43.8%), which supports this notion and is consistent with previous reports, indicating that false-negative PCR results may occur due to an inadequate amount of biopsy tissue. Additionally, surgical specimen samples may contain various components, including normal tissue and various inhibitors of PCR amplification. These inhibitors can be removed by dilution, but this process also reduces the amount of target DNA, resulting in a low possibility of detecting DNA. Therefore, obtaining a pure DNA extent is important; so tissue microdissection to isolate the necrotizing granulomatous inflammatory area can overcome it. According to Ryan et al., 40% of cases that use microdissected tissue detected 155 bp fragments in the IS900 region, while 20% of cases employed entire sections of paraffin-embedded tissue blocks. However, we did not perform the tissue microdissection, so the possibility of detecting TB-DNA becomes lower. Second, low DNA quality can lead to low sensitivity. The extent of formalin penetration and fixation time are important factors when recovering DNA from fixed tissues. A long formalin exposure time will cause DNA fragmentation and decrease the DNA's quality. Studies on the effect of a longer fixation time on DNA amplification products in tissue specimens have revealed that DNA has a greater chance of damage and fragmentation, so shorter DNA fragments are typically extracted from formalin-fixed tissue as time goes on. Successful amplification occurred after 2–24-hour formalin exposure times.

Third, some cases may not have contained the IS6110 element. The target was the IS6110 sequence, which is specific for most MTB complex bacilli (Mycobacterium bacilli, Mycobacterium bovis, Mycobacterium bovis bacillus Calmette-Guerin, Mycobacterium africanum, and Mycobacterium microti). However, the copy number of the sequence inserted into each bacillus genome varied from 0 to 30 depending on the organism. PCR can detect 10 fg or two bacilli in a tissue specimen; however, specimens without the IS6110 element will always be negative.

Table 3. Statistics of each method based on the mycobacterial culture results

| Method   | Culture | No. of cases | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|----------|---------|--------------|----------------|----------------|---------|---------|
| PCR      | (+)     | 46           | 43             | 405            | 51.1    | 86.3    | 51.7    | 86.1    |
|          | (-)     | 44           | 272            |                |         |         |         |         |
| AFB stain| (+)     | 3            | 1              | 91             | 12.5    | 98.5    | 75.0    | 75.9    |
|          | (-)     | 21           | 66             |                |         |         |         |         |
| IGRA     | (+)     | 21           | 30             | 91             | 80.8    | 53.8    | 41.2    | 87.5    |
|          | (-)     | 5            | 35             |                |         |         |         |         |

PPV, positive predictive value; NPV, negative predictive value; PCR, polymerase chain reaction; AFB, acid-fast bacilli; IGRA, tuberculosis-specific antigen-induced interferon gamma release assay.

Table 4. Statistical analysis of PCR-based on the histological findings

| Method                                      | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---------------------------------------------|-----------------|-----------------|---------|---------|
| Necrotizing granuloma (n=131)               | 65.5            | 57.9            | 52.9    | 69.8    |
| Non-necrotizing granuloma (n=61)            | 26.3            | 81.0            | 38.5    | 70.8    |
| Necrosis only (n=38)                        | 66.7            | 91.4            | 40.0    | 97.0    |
| No granuloma and necrosis (n=175)           | 23.1            | 98.8            | 60.0    | 94.2    |

PCR, polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value.

Table 5. Concordance rates between methods

| Method                                      | C coefficient | p-value |
|---------------------------------------------|---------------|---------|
| PCR and culture                             | 0.376         | <.001   |
| PCR and IGRA                                | 0.126         | .034    |
| PCR and AFB stain                           | 0.235         | <.001   |
| PCR and histopathologic features            | 0.442         | <.001   |
| Culture and positive result for both PCR and histopathologic features | 0.288 | <.001 |
| Culture and positive result for PCR or histopathologic features | 0.388 | <.001 |

PCR, polymerase chain reaction; IGRA, tuberculosis-specific antigen-induced interferon gamma release assay; AFB, acid-fast bacilli.

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Furthermore, a false-positive nested PCR result can occur in a tissue specimen. Nested PCR requires additional steps compared to PCR conducted in a single tube, so it is very sensitive even for paraffin-embedded tissue specimens. However, the extra steps may also result in contamination, causing a false-positive result.

The presence of a false-negative culture result can also affect the diagnostic efficiency of PCR. Nested PCR is so sensitive that it can detect residual DNA in a tissue specimen even after medical therapy. Salian et al. suggested that healed TB granulomas can be PCR-positive, even though the culture and AFB staining were negative. Hernandez-Pando et al. also suggested that MTB DNA is located in the macrophages and in other phagocytic cells, which can be important for eliminating latent and persistent bacilli. Among our 315 cases with negative culture results, 45 were PCR-positive and 32 cases showed necrotizing granulomatous inflammation. This finding suggests that the bacteria were not successfully isolated for culture and that additional PCR may help detect the TB bacteria. Miller et al. explained that the higher sensitivity of PCR than culture is due to the increased number of viable organisms, whereas the presence of viable bacteria is crucial for culture. Therefore, they recommended combining bacterial culture and PCR to effectively diagnose TB.

As the diagnostic criteria include the histological features of TB, it is essential to identify necrotizing granulomatous inflammation. Yum and Choi showed that histological features are significantly associated with the TB-positive rate in pleural tissue biopsies (71.4% vs 15.8%). Jambhekar et al. reported a good correlation between typical granulomas and PCR results. According to their findings, specimens with no granuloma have a 20% positive rate, whereas the presence of a granuloma results in 72.5% positivity (84.6% in well-formed granuloma cases). The histological findings were correlated with a higher PCR sensitivity, which increases in cases of necrotizing granulomatous inflammation and decreases in cases of nonspecific inflammation (65.5% vs 23.1%, respectively).

Thus, only one of these features increased the sensitivity. One other study reported that specimens with a granuloma have a 2.8-times higher possibility of detecting bacilli than those without, and our data support this finding (11.6 times, 35.0% vs 3.0%).

Our data showed the highest sensitivity for specimens with necrosis but no granuloma. According to Nopvichai et al., necrotizing non-granulomatous lymphadenitis had an overall PCR-positive rate of 28% (10 of 35) with a sensitivity of 33% compared to the culture results. Therefore, necrotizing specimens without granuloma are typically attributed to TB, so PCR should be performed in these cases. However, our results were limited to three culture-positive cases.

Among the 90 specimens from patients with positive culture results, 13 cases (14.4%) had no histological features of TB, which may have been due to differences between the specimens; however, three cases (1.7%) were PCR-positive. To explain these cases, a patient with TB may have had neither granuloma nor necrosis, or the patients could have had recurrent TB or were taking TB medications, which would suggest that Mycobacterium remain even when no histological changes are present.

Although the PCR sensitivity in selected patients was not high, we found that PCR was the best alternative to culture for detecting TB. The concordance rate between PCR and the histological findings was high, suggesting that typical histological findings can be used to identify Mycobacterium by PCR. In addition, the culture and the combination of PCR and the histological findings showed the highest concordance rate, so we can recommend that the histopathologic findings be added to help detect TB when the PCR result is negative.

Generally, the results of our study indicated that the IGRA was the most sensitive method to diagnose TB. Blood-based in vitro IGRA have been developed as alternatives to the tuberculin skin test for identifying a TB infection. Previous data revealed that the sensitivity and the specificity of IGRA in serum for

### Table 6. PCR statistics values of previous reports

| Reference            | Positive criteria | PCR methods | No. of cases | Type of specimens | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|----------------------|-------------------|-------------|--------------|-------------------|----------------|----------------|---------|---------|
| Park et al.¹⁴        | Culture           | Nested      | 152          | FFPE, lung        | 85             | 99             | 98      | 88      |
| Frevel et al.¹⁵      | Histology, culture, clinical finding | NA          | 229          | FFPE, various organs | 90            | 92             | 81      | 96      |
| Mishra et al.¹⁶      | Culture           | Real time   | 28           | FFPE, gastrointestinal specimens | 96.4          | 100            | 100     | 96      |
| Seo et al.¹⁷        | Culture           | Nested      | 88           | FFPE, various organs | 87.5          | 98.2           | 96.6    | 93.2    |
| Diaz et al.¹⁸       | Clinical and microbiological | Nested      | 43           | FFPE, liver       | 58            | 96             | NA      | NA      |
| Salian et al.¹⁹     | Culture           | NA          | 60           | FFPE, various organs | 100           | 93             | 76.9    | 100     |
| Lee et al.²⁰        | Culture           | Nested      | 129          | FFPE, various organ | 68.3          | 98.5           | NA      | NA      |
| Present study       | Culture           | Nested      | 405          | FFPE, various organ | 51.1          | 86.3           | 51.7    | 86.1    |

PCR, polymerase chain reaction; PPV, positive predictive value; NPV, negative predictive value; FFPE, formalin fixed, paraffin embedded tissue; NA, not available.
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Conflicts of Interest

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

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CD9 Expression in Colorectal Carcinomas and Its Prognostic Significance

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Colorectal cancer (CRC) is ranked as the third most common malignancy by incidence and the fourth most common cause of cancer-specific death worldwide. Although many diagnostic and therapeutic strategies have been developed recently, the mortality rate from CRC remains high. Previous studies have increased our understanding of genetic alterations involved in cancer development and molecular subtypes of CRCs, but accurate prediction of clinical outcomes is still difficult. Therefore, identifying prognostic biomarkers is important for understanding of tumor progression and identifying potential therapeutic targets for CRC.

CD9 is a member of the tetraspanin superfamily that contains four transmembrane domains delimiting three intracellular and two extracellular domains. At least 33 tetraspanin members have been discovered in humans, such as CD9, CD81, CD151, and CD37. These tetraspanin members are involved in many pathophysiologic processes including cellular adhesion, growth, motility, cell-cell fusion in fertilization, signal transduction, and tumor metastasis. The tetraspanins form functional complexes via interaction with various membrane proteins including integrins, epidermal-growth-factor-like growth factors (HB-EGFs), and other tetraspanin proteins, which is known as the "tetraspanin web." Among the tetraspanin members, CD9 is the most common member and is widely expressed in various types of tumor cells as well as normal hematopoietic, smooth muscle, endothelial, and epithelial cells. Many researchers have shown an inverse correlation between CD9 expression and patient survival in breast, stomach, ovarian carcinoma, and malignant melanoma. In vitro experiments manipulating cancer cell lines showed that the overexpression of CD9 in tumor cells or treatment with anti-CD9 monoclonal antibodies (Abs) that augment CD9 function suppresses metastatic potential, migration, and motility of tumor cells in melanoma, lung, and colon cancers. However, evaluation of the specific role of CD9 in tumor-associated immune cells in surrounding stroma has never been carried out. Regarding the tumor microenvironment, tumor-associated immune cells have a significant impact on tumor growth and regression. Therefore, analyzing the prognostic implications of CD9 expression in tumor cells and immune cells is warranted.

In the present study, we immunohistochemically evaluated CD9 expression status and analyzed its prognostic implications.
and its association with various clinicopathological parameters in CRC specimens.

MATERIALS AND METHODS

Patients and specimens
A total of 354 CRC formalin-fixed paraffin-embedded tissues were collected retrospectively from the pathologic archives of Yeungnam University Medical Center, Daegu, Korea. All samples were derived from patients who underwent radical resection and subsequent adjuvant chemotherapy from 1996 to 2000 in our institution. Among these, 49 patients (32 patients who had preoperative neoadjuvant chemotherapy therapy, 16 patients who were diagnosed with stage IV disease, and 1 patient who was diagnosed with stage IV and had preoperative neoadjuvant chemoradiation therapy at the same time) were excluded. Patients’ electronic medical records and pathologic reports were reviewed to obtain information including age, sex, location of tumor, pT/pN categories, presence of lymphatic/venous/perineural invasion, and tumor differentiation. The follow-up period for patients (from the date of surgery to the date of recurrence or the last follow-up) ranged from 4 to 243 months and the average disease-free survival (DFS) was 85 months. The restaging of tumors and histological re-grading were carried out according to the American Joint Committee on Cancer Staging Manual, seventh edition. This study was approved by the Yeungnam University Medical Center Institutional Review Board (No. 2016-07-020).

Tissue microarray and immunohistochemistry
Tissue microarray (TMA) construction was conducted by reviewing hematoxylin and eosin stained slides and selecting an area that had sufficient tumor with no hemorrhage or necrosis. One representative tissue core (2 mm in diameter) was obtained from a donor block and placed in a recipient block using a trephine apparatus. Five cases of non-neoplastic colorectal (Fig. 1A), renal, hepatic parenchymal tissues and three cases of non-neoplastic gastric parenchymal tissues were included in each TMA. Seven tissue array blocks were prepared for 305 cases. Immunohistochemical (IHC) staining with rabbit monoclonal Abs against CD9 (1:1,200, EPR2949; Abcam, Cambridge, UK) was performed on each TMA block using a BenchMark XT immunostainer (Ventana Medical System, Tucson, AZ, USA) following the manufacturer’s protocol. Immunoreactivity of CD9 expression was assessed in tumor cells (T-CD9) and immune cells (I-CD9) in stroma semi-quantitatively. In cases of T-CD9, both intensity and extent of membranous-to-cytoplasmic staining were evaluated. The intensity was initially scored on a scale of 0 to 3: negative (0), weak positive (1+), moderately positive (2+), and strongly positive (3+) (Fig. 1B–D). The extent of staining was categorized into five groups according to the percentages of positively stained cells: 0% (0), ≤25% (1+), >25 and ≤50% (2+), >50 and ≤75% (3+), and >75% (4+). The final T-CD9 score was determined by multiplying the extent and intensity scores and recording the final score from 0 to 12. The T-CD9 immunoreactivity score was then dichotomized as low (score, 0 to 3) or high (score, 4 to 12). In cases of I-CD9, the percentage of stained immune cells was estimated on a 0, 1+, 2+, and 3+ scale: 0% (0), ≤25% (1+), >25 and ≤50% (2+), and >50% (3+) (Fig. 2). The cases of I-CD9 score 0 were classified as low, and when the score was 1 to 3, they were classified as high. All IHC staining assessments were performed independently by two pathologists (K.-J.K. and Y.K.B.), who were blinded to the clinicopathological information. Conflicting evaluations between the pathologists were discussed and a consensus was achieved.

Statistical analysis
IBM SPSS ver. 20.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Correlation between the categorical variables was analyzed using Pearson’s chi-square test or Fisher exact test. DFS was defined as the period from the date of primary radical resection to the date of tumor recurrence, metastasis, death, or the last follow-up. DFS was assessed by the Kaplan-Meier method with the log-rank test, and multivariate analysis with the Cox regression model was used to adjust variables that had been statistically significant for DFS in univariate analysis. All statistical analyses were two-sided, and statistical significance was considered to be p < 0.05.

RESULTS

CD9 expression status in CRC
Five normal sample of colorectal tissue and 305 CRC samples were examined to identify CD9 expression. Of the 305 CRC samples, one case T-CD9 sample was excluded from evaluation because repeated sectioning of the TMA block resulted in very few tumor cells remaining for evaluation. As a result, 304 cases of T-CD9 and 305 cases of I-CD9 were analyzed. Among the five samples of non-neoplastic colorectal mucosae, the expression of CD-9 was negative or weakly positive in almost all epithelial cells (Fig. 1A). These weakly positive cells showed similar intensity and were classified into the low expression group. On the other hand, more than 50% of stained stromal immune cells in non-
neoplastic colorectal tissues exhibited strong expression of CD9 throughout the entire samples and were classified as the high expression group. Meanwhile, tumor cells and surrounding stromal immune cells showed variable expression patterns. T-CD9 (+) was detected in 175 of 304 CRGs (57.6%), and I-CD9 (+) was detected in 265 of 305 cases (86.9%). One hundred fifty-four cases (50.7%) showed both T-CD9 (+) and I-CD9 (+) and 19 cases (6.3%) exhibited both T-CD9 (–) and I-CD9 (–).

Characteristics of a T-CD9 subset of CRC

No statistically significant differences were noted in clinicopathological parameters including age, sex, stage, gross type, location, pT/pN categories, or frequency of angiolymphatic and perineural invasion between the T-CD9 (+) and T-CD9 (–) groups (Table 1). In Kaplan-Meier survival analysis, the T-CD9 (+) group showed a tendency for better DFS than the T-CD9 (–) group, but statistical significance was not reached (p = .057) (Fig. 3A). We additionally subdivided cases into two groups according to tumor location: right-sided tumors (n = 89) and left-sided tumors (n = 215). Based on the previous literature,18 CRCs were classified into right- and left-sided tumors relative to the splenic flexure. In other words, tumors arising proximal to the splenic flexure (cecum, ascending colon, and transverse colon) are considered right-sided, and tumors distal to the splenic flexure (descending colon, sigmoid colon, and rectum) are considered left-sided. In left-sided tumors, DFS was significantly better in the T-CD9 (+) group compared to the T-CD9 (–) group (p = .021) (Fig. 3B). However, in right-sided tumors, no significant difference was observed between the two groups (p = .453) (Fig. 3C). Multivariate analysis including stage, lymphatic invasion, vascular invasion, perineural invasion and T-CD9—which were significant prognostic factors in univariate analysis—revealed that the stage (p = .002) was still an independent prognostic predictor for DFS, whereas T-CD9 expression failed to correlate with prognosis, with no difference between T-CD9 (+) and T-CD9 (–) groups in patients with left-sided CRC (p = .167) (Table 2).

**Fig. 1.** Immunohistochemical expression of CD9 in non-neoplastic colorectal mucosa (A) and tumor cells exhibiting intensity score 0 (B), intensity score 1 (C), and intensity score 3 (D).
Characteristics of an I-CD9 subset of CRC

I-CD9 (+) CRCs significantly correlated with well/moderately differentiation \((p = .014)\) compared to I-CD9 (–) CRCs. However, no significant associations were found for other parameters including age, sex, stage, gross type, location, pT/pN categories, or frequency of angiolymphatic and perineural invasion (Table 1). In Kaplan-Meier survival analysis, the I-CD9 (+) group had a tendency towards worse DFS compared to the I-CD9 (–) group, but no significant difference was found \((p = .156)\) (Fig. 3D). When the cases were subdivided into left- and right-sided CRC groups, I-CD9 (–) suggested a potential protective role in left-sided tumors \((p = .235)\), but statistical significance was not reached (Fig. 3E). In right-sided tumors, no statistical significance was observed between the two groups \((p = .645)\) (Fig. 3F).

Characteristics of a combined T-CD9/I-CD9 subset of CRC

Kaplan-Meier survival analysis found the longest DFS for patients in the T-CD9 (+)/I-CD9 (–) group, whereas the T-CD9 (–)/I-CD9 (+) group had the shortest DFS, but statistical significance was not reached \((p = .054)\) (Fig. 4A). When the patients were divided into left- and right-sided CRC groups, increased survival differences between subgroups was observed in left-sided tumors \((p = .030)\) (Fig. 4B). In right-sided tumors, no significant difference in DFS was observed \((p = .957)\) (Fig. 4C). In multivariate analysis using a Cox proportional hazards model, combined T-CD9/I-CD9 expression was not an independent prognostic factor of DFS in left-sided CRCs \((p = .199)\) (Table 2).

DISCUSSION

Many previous in vitro and in vivo models of cancer cell lines have contributed to our understanding of the role of CD9 and the associated functional complexes with other members of the tetraspanin family and various integrins.\textsuperscript{14-16,19} High expression of CD9 in tumor cells has been associated with favorable prognosis, reduced metastatic potential, and cancer growth in various tumor

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{immunohistochemical_expression_of_cd9_in_immune_cells_in_colorectal_carcinomas.png}
\caption{Immunohistochemical expression of CD9 in immune cells in colorectal carcinomas. Representative case exhibiting score 0 (A), score 1 (B), score 2 (C), and score 3 (D).}
\end{figure}
types, including lung, breast, prostate, stomach, pancreas, and colon carcinoma. Miyake et al. demonstrated that injection of CD9-transfected melanoma cells suppressed lung metastasis in a rodent model and Ovalle et al. showed that ectopic CD9 expression in a colon cancer cell line resulted in inhibition of cell growth. In addition, Mori et al. stated that reduced expression of CD9 by reverse transcription polymerase chain reaction is linked to poor prognosis in colon cancer. Our data demonstrated that patients with high expression of CD9 in tumor cells (T-CD9 [+]) tended to have a longer DFS, although the difference was not statistically significant ($p = .057$).

However, studies have yielded controversial results about CD9 expression in non-neoplastic colorectal cells. Mori et al. demonstrated that CD9 mRNA is consistently expressed in non-neoplastic colonic tissues adjacent to tumors and its expression level was higher in normal colonic tissues than in tumors in six normal/tumor paired samples using northern blot analysis. Okochi et al. also showed that strong CD9 expression was detected in normal colonic epithelium by IHC. On the contrary, negative or weakly positive expression of CD9 was observed in

| Table 1. Association of clinicopathologic features with T-CD9 and I-CD9 |
|-----------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Variable                    | CD9 expression in tumor cells (T-CD9)* | CD9 expression in immune cells (I-CD9) |
|                             | No. of cases | Low | High | p-value | No. of cases | Low | High | p-value |
| Age (yr)                    | .449         | .496 |
| ≤60                         | 204          | 83 (64.3) | 121 (69.1) | 205 | 25 (62.5) | 180 (67.9) |
| >60                         | 100          | 46 (35.7) | 54 (30.9) | 100 | 15 (37.5) | 85 (32.1) |
| Sex                         | .175         | .581 |
| Male                        | 163          | 75 (58.1) | 88 (50.3) | 163 | 23 (57.5) | 140 (62.8) |
| Female                      | 141          | 54 (41.9) | 87 (49.7) | 142 | 17 (42.5) | 125 (47.2) |
| Tumor location              | .224         | .385 |
| Right                       | 89           | 33 (25.6) | 56 (32.0) | 89 | 14 (35.0) | 75 (28.3) |
| Left                        | 215          | 96 (74.4) | 119 (68.0) | 216 | 26 (65.0) | 190 (71.7) |
| Gross type                  | .775         | .632 |
| Fungating                   | 137          | 61 (48.0) | 76 (46.3) | 137 | 16 (43.2) | 121 (47.5) |
| Infiltrative                | 154          | 66 (52.0) | 88 (53.7) | 155 | 21 (56.8) | 134 (52.5) |
| AJCC TNM stage              | .938         | .156 |
| Stage I                     | 32           | 13 (10.1) | 19 (10.8) | 33 | 1 (2.5) | 32 (12.1) |
| Stage II                    | 134          | 56 (43.4) | 78 (44.6) | 134 | 21 (52.5) | 113 (42.6) |
| Stage III                   | 138          | 60 (46.5) | 78 (44.6) | 138 | 18 (45.0) | 120 (45.3) |
| Tumor depth (pT)            | .909         | .137 |
| pT1                         | 9            | 4 (3.1) | 5 (2.8) | 9 | 0 | 9 (3.4) |
| pT2                         | 30           | 11 (8.5) | 19 (10.9) | 31 | 1 (2.5) | 30 (11.3) |
| pT3                         | 233          | 101 (78.3) | 132 (75.4) | 233 | 36 (90.0) | 197 (74.3) |
| pT4                         | 32           | 13 (10.1) | 19 (10.9) | 32 | 3 (7.5) | 29 (11.0) |
| LN metastasis (pN)          | .884         | .935 |
| pN0                         | 167          | 69 (53.5) | 98 (56.0) | 168 | 22 (55.0) | 146 (65.1) |
| pN1                         | 86           | 37 (28.7) | 49 (28.0) | 86 | 12 (30.0) | 74 (27.9) |
| pN2                         | 51           | 23 (17.8) | 28 (16.0) | 51 | 6 (15.0) | 45 (17.0) |
| Tumor differentiation       | .734         | .014 |
| WD/MD                       | 261          | 110 (85.9) | 151 (87.3) | 262 | 29 (74.4) | 233 (88.6) |
| PD/Other                    | 40           | 18 (14.1) | 22 (12.7) | 40 | 10 (25.6) | 30 (11.4) |
| Lymphatic invasion          | .394         | .361 |
| Absent                      | 192          | 77 (67.5) | 115 (72.3) | 193 | 21 (63.6) | 172 (71.4) |
| Present                     | 81           | 37 (32.5) | 44 (27.7) | 81 | 12 (36.4) | 69 (28.6) |
| Venous invasion             | .467         | .300 |
| Absent                      | 208          | 85 (74.6) | 123 (78.3) | 209 | 23 (69.7) | 186 (77.8) |
| Present                     | 63           | 29 (25.4) | 34 (21.7) | 63 | 10 (30.3) | 53 (22.2) |
| Perineural invasion         | .553         | .856 |
| Absent                      | 195          | 84 (75.0) | 111 (78.2) | 196 | 25 (78.1) | 171 (76.7) |
| Present                     | 59           | 28 (25.0) | 31 (21.8) | 59 | 7 (21.9) | 52 (23.3) |

Values are presented as number (%).
AJCC, American Joint Committee on Cancer; LN, lymph node; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.
*One case is excluded from this statistical analysis due to unavailable TMA data; Only patients with available clinicopathological data are included.

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Fig. 3. Kaplan-Meier survival analysis with log-rank test of CD9 expression. (A) Survival curves of the T-CD9 (−) versus T-CD9 (+) in all cases. (B) Survival curves of the T-CD9 (−) versus T-CD9 (+) in left-sided tumors. (C) Survival curves of the T-CD9 (−) versus T-CD9 (+) in right-sided tumors. (D) Survival curves of the I-CD9 (−) versus I-CD9 (+) in all cases. (E) Survival curves of the I-CD9 (−) versus I-CD9 (+) in left-sided tumors. (F) Survival curves of the I-CD9 (−) versus I-CD9 (+) in right-sided tumors. T-CD9, CD9 expression in tumor cells; I-CD9, CD9 expression in immune cells.
**Table 2. Multivariate survival analysis of factors associated with disease-free survival in left sided CRCs**

| Parameter                          | Univariate analysis | Multivariate analysis I | Multivariate analysis II |
|------------------------------------|---------------------|-------------------------|--------------------------|
|                                   | Hazard ratio (95% confidence interval, mo) | p-value | Hazard ratio (95% confidence interval, mo) | p-value | Hazard ratio (95% confidence interval, mo) | p-value |
| AJCC stage                         | <.001               | .002                    | .001                     |
| Stage I/II                         | Reference           | Reference               | Reference                |
| Stage III                          | 3.831 (2.210-6.644) | 2.727 (1.448-5.136)     | 2.807 (1.495-5.272)     |
| Lymphatic invasion                 | <.001               | .121                    | .101                     |
| Absent                             | Reference           | Reference               | Reference                |
| Present                            | 2.852 (1.647-4.338) | 1.794 (0.858-3.755)     | 1.843 (0.887-3.831)     |
| Vascular invasion                  | .001                | .461                    | .485                     |
| Absent                             | Reference           | Reference               | Reference                |
| Present                            | 2.552 (1.438-4.529) | 1.330 (0.623-2.838)     | 1.308 (0.616-2.777)     |
| Perineural invasion                | .006                | .163                    | .143                     |
| Absent                             | Reference           | Reference               | Reference                |
| Present                            | 2.202 (1.248-3.887) | 1.540 (0.840-2.824)     | 1.577 (0.857-2.905)     |
| T-CD9                              | .023                | .167                    | Not included             |
| Low                                | Reference           | Reference               | –                        |
| High                               | 0.558 (0.337-0.922) | 0.669 (0.379-1.183)     | –                        |
| T-CD9/I-CD9                        | Not included        | Not included            | .199                     |
| T-CD9 (+)/I-CD9 (-)                | Reference           | –                       | Reference                |
| T-CD9 (+)/I-CD9 (+) and T-CD9 (-)/I-CD9 (-) | 3.591 (0.489-26.351) | .209 | – | – | 3.136 (0.419-23.459) | .266 |
| T-CD9 (-)/I-CD9 (+)                | 5.983 (0.816-43.860) | .078 | – | – | 4.492 (0.600-33.611) | .143 |

CRC, colorectal carcinoma; AJCC, American Joint Committee on Cancer; T-CD9, CD9 expression in tumor cells; I-CD9, CD9 expression in immune cells.
normal colorectal epithelial cells in our study. These contradictory finding of the non-neoplastic CD9 expression may be attributed to differing experimental techniques such as northern blot analysis and IHC, as well as variation in the different types of CD9 antibodies. Furthermore, our data has limitation in this regard, because very few normal colorectal tissue samples (n = 5) were included, and pairing of normal and tumor samples has not been carried out. Further investigation using extended cases of paired samples are needed for clarification.

Several mechanisms have been suggested to explain the tumor suppressor function of CD9. In conjunction with EGFR, CD9 has been shown to induce apoptosis via restricted activation of p46 Shc isoforms or the c-Jun N-terminal kinase/stress-activated protein kinase and p38 mitogen-activated protein kinase pathways. By forming a functional complex with integrin β1, CD9 suppresses cell motility and enhances cell integrity and adhesion. Furthermore, CD9 may hinder cell proliferation through internalization of EGFR, resulting in attenuation of EGF-EGFR induced signals. Notably, the protective prognostic role of T-CD9 on DFS was demonstrated in the relatively homogenous left-sided CRC group in this study with statistical significance (p = .021). However, in the right-sided tumor group, no significance of T-CD9 was observed in DFS, but this finding was considered to have limited reliability due to the small number of cases (n = 89). The prognostic effect of T-CD9 is speculated to be attributed to different molecular carcinogenic mechanisms between right- and left-sided CRCs. In left-sided CRCs, the majority of tumors develop through a chromosomal instability pathway known as the adenoma-carcinoma sequence. In this pathway, progression to adenoma-carcinoma from normal mucosa is established by accumulation of various genomic changes including activation of proto-oncogenes such as KRAS and inactivation of tumor suppressor genes such as APC and TP53. Meanwhile, a large proportion of tumors with microsatellite unstable (microsatellite instability–unstable) subtypes arise predominantly in the right side of the colon. They are caused by genetic and epigenetic alterations of mismatch repair genes and consequently exhibit a hypermutated phenotype. Serrated morphology tumors and CpG island methylator phenotypes are seen more frequently in right-sided tumors than in left-sided CRCs. These findings suggest that even in the same tumor, the prognostic implication of CD9 can be different depending on the underlying molecular mechanisms of carcinogenesis. Extended study is required to verify the hypothesis presented above and the location based variation of CD9 roles in tumor progression.

Tumor-infiltrating immune cells are thought to play a substantial role in shaping the microenvironment depending on various factors such as cytokines and chemokines, towards either immunostimulatory antitumor conditions or immunoregulatory tumor promoting milieu. In survival analysis, I-CD9 (+) showed a tendency to be associated with a high recurrence rate in left-sided CRCs (p = .156) and this finding is contrary to the case of T-CD9. In a previous study, Erovic et al. described that CD9-expressing tumor-infiltrating lymphocytes in squamous cell carcinoma of the head and neck. Bruno et al. demonstrated that CD9-expressing tumor-infiltrating immune cells, especially tumor-associated natural killer cells, are polarized to have pro-angiogenic and pro-tumoral phenotypes. These findings are consistent with our results regarding I-CD9. To the best of our knowledge, this is the first report to identify a differential prognostic role of CD9 expression in tumor cells and immune cells in CRCs.

Murayama et al. stated that a therapeutic agent targeting
tetraspanin, especially CD9, using monoclonal Abs such as ALB6 and PAIN13, might be beneficial for cancer patients. However, several researchers have suggested the contradictory finding of T-CD9 in various cancer types including multiple myeloma, fibrosarcoma, and even in gastric carcinoma, showing that CD9 expression of tumor cells has a positive influence on tumor proliferation and invasion by enhancing HB-EGF/EGFR interaction or metalloproteinases-2 secretion. In addition, our study also demonstrated an opposite effect of T-CD9 and I-CD9 on tumor progression. Therefore, future studies should focus on the varying mechanisms and the therapeutic response of I-CD9 and T-CD9 in CRCs.

In combined survival analysis of T-CD9 and I-CD9, tumors with high T-CD9 expression plus low I-CD9 expression showed a tendency to have a longer DFS compared with (1) tumors with concurrent high or low expression of T-CD9 and I-CD9, or (2) tumors with low T-CD9 expression plus high I-CD9 expression (p = .054), and a similar result was found in left-sided CRCs with statistical significance (p = .030). These findings suggested that the combined evaluation of T-CD9 and I-CD9 is required to determine the comprehensive prognostic effect of CD9 in CRCs.

In conclusion, high expression of CD9 in tumor cells tends to be inversely related to tumor recurrence, especially in left-sided CRCs. However, a different tendency on DFS was demonstrated in immune cells regardless of tumor location. Additional studies are necessary, with an emphasis on differential expression of CD9 in tumor cells and immune cells.

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

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A Case of Malignant PEComa of the Uterus Associated with Intramural Leiomyoma and Endometrial Carcinoma

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Perivascular epithelioid cell tumors (PEComas) refers to a family of mesenchymal neoplasms composed of angiomyolipomas, clear cell “sugar” tumors of the lung, and lymphangioleiomyomatoses. These tumors have a distinctive and common component of perivascular epithelioid cells that show an association with blood vessel walls and immunohistochemically display myo-melanocytic differentiation. The unique neoplasms have been shown to have an expanded range through a variety of case reports, including visceral, intra-abdominal, soft tissue, and bone tumors. The retroperitoneum, abdominopelvic region, and uterus have been reported to be the most common sites. Most PEComas follow a benign course. However, reports of malignant PEComas are increasing. Many papers have described uterine PEComas, but to our knowledge, there have not yet been any reports of a malignant PEComa arising concomitant with another epithelial tumor and mesenchymal tumor. We report herein the case of a 67-year-old woman who experienced a malignant uterine PEComa infiltrating a preexisting intramural leiomyoma with synchronous well differentiated endometrial carcinoma and multiple liver and lung metastases.

Key Words: Immunohistochemistry; Perivascular epithelioid cell neoplasms; Uterus

CASE REPORT

A 67-year-old post-menopausal woman visited the clinic due to vaginal bleeding. Ultrasonography revealed one myometrial mass and one endometrial lesion. Levels of serum tumor markers, including carbohydrate antigen 125 and carbohydrate antigen 19-9, were normal. Curettage of the endometrial lesion showed endometrioid adenocarcinoma, the International Federation of Gynecology and Obstetrics (FIGO) G1. Imaging studies revealed multiple suspicious metastatic lesions in the liver and lung. A total abdominal hysterectomy and bilateral salpingo-oophorectomy were performed.

There was an ill-demarcated grayish mass, measuring $4 \times 3.7 \times 3.5$ cm, on the anterior wall of the endometrium considered to be endometrial origin. There was another well-demarcated heterogeneous white to yellowish partially hemorrhagic and fibrotic mass, measuring $6 \times 5 \times 4.4$ cm, on the posterior wall of the myometrium. The cut surface of the mass showed an ill-demarcated yellowish necrotic lesion, enclosed by a whitish fibrotic area. It measured $3.7 \times 2.5 \times 2.5$ cm (Fig. 1).

The endometrial lesion had the histologic appearance of endometrioid carcinoma, the same as the previous curettage (Fig. 2A). It invaded the myometrium $16$ mm of the total $20$ mm thickness.

The histologic findings of the myometrial mass were characterized by infiltration of spindle to ovoid cells into the surrounding leiomyoma. The infiltrating cells showed clear cytoplasm and elongated nuclei with cytological atypia. Frequent mitotic activity was encountered in up to $14/50$ high-power fields (HPF). Coagulative tumor cell necrosis was present (Fig. 2B, C).

Immunohistochemical staining was performed on the myometrial lesion for CD10, caldesmon, desmin, α-smooth muscle...
actin (SMA), human melanoma black 45 (HMB-45), Melan-A, transcription factor E3 (TFE3), and Ki-67. The infiltrating tumor cells were positive for SMA, HMB-45, and TFE3 with a low Ki-67 index of about 5%, but they were negative for CD10, caldesmon, and desmin (Fig. 3A, B). In contrast, the surrounding leiomyoma showed positivity for caldesmon and desmin (Fig. 3C).

**DISCUSSION**

PEComas, which have no known normal tissue counterpart, are unique in that they react immunohistochemically for both melanocytic and myoid markers. Folpe et al. reviewed 26 cases of PEComas of soft tissue and gynecologic origin. According to that report, all cases demonstrated expression of at least one of the melanocytic markers (HMB-45, Melan-A, and microphthalmia transcription factor), and HMB-45 showed the highest rate of positivity at 92%. They also showed positivity for myoid markers with SMA (80%) and desmin (36%).

Our case demonstrated positivity for both HMB-45 and Melan-A, and melanin pigments were observed in some cells. Among the myoid markers, our case was positive for SMA and negative for desmin and caldesmon. The existing leiomyoma was positive for desmin and caldesmon, so the PEComa was clearly defined from the large preexisting leiomyoma at the peripheral portion of the bulky mass.

We reviewed all case series published in English available in PubMed that included more than three cases of PEComa in the female genital tract, for a total of 63 cases. The results (Table 1) show that all (100%) of the tested cases showed positivity for HMB-45, and 52.8% were positive for Melan-A. Myoid markers including SMA, caldesmon, and desmin were positive in 74.5%, 66.0%, and 94.4% of tested cases, respectively. CD10 immunostaining was performed in 23 cases and showed positivity in six cases (26.1%). Five out of 13 cases (38.5%) were positive for TFE3. These results confirm the diagnostic value of immunohistochemistry profiling in uterine PEComa, especially the use of melanocytic and myoid markers, due to their high rates of positivity.
The differential diagnosis of uterine PEComa includes uterine smooth muscle tumor, endometrial stromal tumor, gastrointestinal stromal tumor (GIST) with secondary involvement of the uterus, and other sarcomas such as alveolar soft part sarcoma (ASPS). Paraganglioma, metastatic melanoma/clear cell sarcoma of soft part, and rarely, carcinoma should also be considered in the differential diagnosis due to their epithelioid cytomorphology. Immunohistochemical staining may be helpful in the differential diagnosis. HMB-45 positivity enables a differential diagnosis of PEComa from uterine smooth muscle tumor, endometrial stromal...
sarcoma, and ASPS. There was a report of late pulmonary and renal metastatic PEComas with initial misdiagnosis as uterine leiomyosarcomas. CD10, which usually shows diffuse and strong immunoreactivity in endometrial stromal tumors, may be helpful in the differential diagnosis because 25% of uterine PEComas are reported to be positive for CD10. The exclusion of GIST from PEComa may be possible on the basis of CD34 staining, as well as c-Kit positivity and melanocytic marker negativity in GISTs. Metastatic melanoma and/or clear cell sarcoma can be distinguished from PEComa by the strong S-100 protein immunoreactivity of the former and their muscle marker negativity.

PEComas harboring TFE3 gene fusion are described in several reports. TFE3 is a member of the MiT family of transcription factors, and TFE3 gene fusions have been found in some neoplasms such as ASPS and a subset group of renal cell carcinoma. Our case also showed strong TFE3 immunoreactivity. In PEComa, the group harboring TFE3 gene fusion is reported to be more common in young patients, to be unrelated to tuberous sclerosis, to show alveolar architecture, and to have more epithelioid than spindle cell cytology and minimal immunoreactivity for myocytic markers. Because of the small number of cases, however, it is difficult to define these cases as a single distinct group showing specific clinical characteristics compared with conventional PEComas. More research and case studies are required.

There was a report of a concomitant PEComa and an endometrioid carcinoma with synchronous/metastatic bilateral ovary carcinomas and uterine leiomyoma. The case had a finding common to ours in that there was a well differentiated endometrial carcinoma involving a depth of more than half of the myometrium with a synchronous separate PEComa. In that case, however, the pathologic examination revealed benign features of PEComa and it presented as a subserosal mass, unlike our case, which presented as a protruding endometrial mass and showed malignant features.

Criteria for malignancy of PEComa of the female genital tract are currently not clearly defined due to insufficient case studies. According to criteria that are currently accepted, PEComas are classified into three categories: benign, uncertain malignant potential, and malignant. Benign is defined to exhibit the following gross or histologic features: gross size < 5 cm, non-infiltrative growth, non-high-grade nuclear features, no necrosis or vascular invasion, and a mitotic rate < 1/50 HPF. Tumors of uncertain malignant potential are defined as corresponding to one or more of the following features: nuclear pleomorphism or multinucleated giant cells, or gross size > 5 cm regardless of cellular features. Satisfying the malignant category refers to cases which show two or more of the following features: gross size > 5 cm, infiltrative growth, high-grade nuclear features, necrosis, vascular invasion, or a mitotic index ≥ 1/50 HPF. According to the largest single series of PEComas of gynecologic origin, the current classification system was very specific and sensitive for the diagnosis of malignant PEComa in the gynecologic tract. The most common solid organ metastatic site according to the study was the lung. Multiple metastases were observed in the lung and liver in our case.

Conflicts of Interest

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

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**A Case of Giant Colonic Muco-submucosal Elongated Polyps Associated with Intussusception**

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**Key Words:** Non-neoplastic colorectal polyps; Colonic muco-submucosal elongated polyp; Intussusception

The term polyp in the gastrointestinal tract is a nonspecific clinical term, broadly utilized when referring to any localized elevation above the surrounding mucosa regardless of its histologic nature, and can be categorized as neoplastic and non-neoplastic polyps. The latter are traditionally classified as hyperplastic, inflammatory, and hamartomatous polyps. Daniels and Montgomery, however, differently subclassified the non-neoplastic polyps in the colorectum into mucosal prolapse, incidental stromal polyps and polyps associated with or without systemic diseases. As such variations in classification, currently there is no clear cut consensus regarding the most ideal or clinically significant classification scheme for the various non-neoplastic colorectal polyps.

Recently, we experienced a large non-neoplastic colorectal polyp which did not match the traditional classification of non-neoplastic colorectal polyps, in addition to being associated with intussusception. In this report, we present a case of a giant variant of the newly classified non-neoplastic colorectal polyp, colonic muco-submucosal elongated polyp (CMSEP), associated with intussusception in a 48-year-old woman, with a review of the literature.

Colonic muco-submucosal elongated polyp (CMSEP), a newly categorized non-neoplastic colorectal polyp, is a pedunculated and elongated polyp composed of normal mucosal and submucosal layers without any proper muscle layer. We herein report a giant variant of CMSEP associated with intussusception in the rectosigmoid colon, with a review of the literature. A 48-year-old woman underwent a laparoscopic low anterior resection due to multiple large submucosal polypoid masses associated with intussusception. Grossly, the colonic masses were multiple pedunculated polyps with a long stalk and branches ranging in size from a few millimeters to 14.0 cm in length. Microscopically, there was no evidence of hyperplasia, atypia, or active inflammation in the mucosa. The submucosal layers were composed of edematous and fibrotic stroma with fat tissue, dilated vessels, and lymphoid follicles.

**CASE REPORT**

A 48-year-old, previously healthy female patient visited the emergency department with initial presentation of intermittent abdominal pain and anal bleeding. The patient explained that the symptom had first begun 4 to 5 years ago. Under the clinical impression of hemorrhoids, the patient underwent a hemorrhoidectomy. The patient had no particular family history, changing bowel habits or mucoid diarrhea. An abdominal computed tomography (CT) was conducted 2 weeks later as a part of the evaluation process, which revealed a colocolic intussusception from the sigmoid colon to the distal rectum. In addition, prominent mucosal folds were observed without any ischemic change or pericolic abnormality. Following the CT scan, a barium enema revealed a large movable and compressible submucosal mass in the distal portion of the sigmoid colon (Fig. 1). Multiple similar yet smaller lesions were also present near the main mass in the sigmoid colon. The intussusception spontaneously regressed without any complications. Colonoscopy revealed several large polypoid masses in the rectosigmoid colon and multiple biopsies were obtained. The biopsied specimens were diagnosed as chronic...
Fig. 1. Barium enema (A) and abdominal computed tomography (B) of colonic muco-submucosal elongated polyp reveal prominent mucosal and submucosal folds (arrow) associated with intussusception (asterisk) in rectosigmoid colon.

Fig. 2. Colonoscopy, gross and microscopic findings of colonic muco-submucosal elongated polyp. (A) Colonoscopy reveals multiple, large, movable, pedunculated, and sessile polyps from descending to sigmoid colon. (B) The polypoid masses show large, tree-like branching mucosal folds with elongated and pedunculated stalks with a broad base. (C) The polyps have long, elongated, and loose submucosal elongated stalks without cytological atypia and active inflammation. (D) α-Smooth muscle actin immunohistochemical stain show prominent smooth muscle of the muscularis mucosae.
nonspecific inflammation without any definite submucosal mass-like lesions. Due to the presence of such large lesions, the patient underwent a laparoscopic low anterior resection in which a 21.5-cm segment of colon was resected.

The surgical specimen revealed multiple, large, tree-like branch polypoid masses, with elongated or pedunculated stalks or a wide broad base, measuring up to \(14.0 \times 5.0 \times 4.5\) cm in dimensions of the largest polypoid mass (Fig. 2A). Upon microscopic examination of the lesions, most of the polyps had long, elongated, and loose or fibrotic submucosal elongated stalks, while some were sessile. There was neither prominent cytologic atypia nor active inflammation in the mucosa. There was also proliferation of mature fat tissue, lymphoid follicles, multiple dilated blood vessels, and hypertrophied smooth muscle of the muscularis mucosae (Fig. 2B). Immunohistochemical staining with \(\alpha\)-smooth muscle actin revealed hypertrophied smooth muscle of the muscularis mucosae (Fig. 2C). However, there was no tree like proliferation of the muscularis mucosae as in Peutz-Jeghers polyps. Staining with other antibodies, such as anaplastic lymphoma kinase, c-Kit, \(\beta\)-catenin, CD34, and S-100 protein, showed negative findings in all.

**DISCUSSION**

The newly categorized non-neoplastic colorectal polyp, CMSEP, was first described by Matake et al.\(^1\) in 1998. Since its first appearance in the literature, there have been various reports of such polyps, although mostly from Japanese institutions.\(^1\)\(^{\text{-}11}\)

The characteristic endoscopic feature is the “worm-like” appearance. Histologically, the polyps are covered by normal mucosa and a loose to dense submucosal layer containing a variably prominent mixture of blood vessels and lymphatics, in the absence of significant active inflammation. Such polyps have been reported in all parts of the large intestines, as well as in the small intestines.\(^3\)\(^{\text{-}}\)\(^{\text{12}}\)\(^{\text{-}}\)\(^{\text{17}}\) Overall, the histologic findings of our case are similar to those of previously reported cases, and our case meets the suggested criteria for the diagnosis of CMSEP by Alizart et al.\(^12\) and Tan et al.\(^16\)

In the literature to date, a total of 48 cases of CMSEPs have been reported (Table 1).\(^3\)\(^{\text{-}18}\) Based on the review of these reports, there seems to be a slight predilection for males (male:female = 1.4:1). All but one of these cases\(^12\) were a single polyp occurring in the colon, and the sigmoid colon was the most common site.\(^2\)\(^{\text{-}}\)\(^{\text{12}}\)\(^{\text{13}}\) Yet, our case is unique in that there was the additional presentation of intussusception and in that the polyps were multiple and much larger in size compared to the majority of the previously reported cases. Furthermore, there has been no report to date of such large polyps in the rectosigmoid colon displaying branching architecture as in our case. The mean size of the reported polyps in the sigmoid colon and rectum was 2.44 cm and 1.12 cm, respectively, which is much smaller compared to the polyps in this report that measured up to 16.0 cm in largest dimensions. Despite the multiplicity and large size, the histologic features of our case match the descriptions of CMSEPs.

Many differential diagnoses must be considered in the diagnosis of CMSEP. The first differential diagnosis includes mucosal

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**Table 1. Review of the literature for CMSEPs**

| Reference (published year) | Country | Sex (M:F) | Age (mean, yr) | Site (number of polyps) | Polyp size (length, cm) |
|---------------------------|---------|-----------|----------------|-------------------------|-------------------------|
| Ivuko\J et al.\(^{11}\) (2015) | Croatia | M | 82 | SC (1) | 2.4 |
| Tan et al.\(^\text{et al}\) (2013) | Singapore | 1:2 | 66.3 | SI (2), DC (1) | 1.4–4.0 |
| Shin et al.\(^\text{et al}\) (2012) | Korea | M | 54 | AC (1) | 3.5 |
| Nishimura et al.\(^\text{et al}\) (2012) | Japan | F | 70 | SI (1) | NS |
| Hirasaki et al.\(^\text{et al}\) (2011) | Japan | M | 79 | SC (1) | 0.7 |
| Ambrosio et al.\(^\text{et al}\) (2011) | Italy | F | 77 | SC (1) | 7.0 |
| Alizart et al.\(^\text{et al}\) (2011) | Australia | 7:4 | 57.6 | SC (6), TC (2), AC (1), DC (1) | 1.0–15.0 |
| Tozawa et al.\(^\text{et al}\) (2009) | Japan | M | 31 | DC (1) | 2.0 |
| Kume et al.\(^\text{et al}\) (2009) | Japan | M | 66 | TC (1) | 4.0 |
| Kanazawa et al.\(^\text{et al}\) (2006) | Japan | M | 53 | AC (1) | 5.0 |
| Akahoshi et al.\(^\text{et al}\) (2003) | Japan | M | 63.6 | SC (2), AC (2), R (3) | 1.0–4.0 |
| Yamamoto et al.\(^\text{et al}\) (2004) | Japan | M | 73 | DC (1) | NS |
| Lee et al.\(^\text{et al}\) (2003) | Korea | M | 44 | AC (1) | 6.0 |
| Takahashi et al.\(^\text{et al}\) (2002) | Japan | M | 52 | TC (1) | 1.0 |
| Domoto et al.\(^\text{et al}\) (1998) | Japan | F | 55 | DC (1) | 3.0 |
| Matake et al.\(^\text{et al}\) (1998) | Japan | 6:9 | 58.3 | TC (6), AC (4), DC (2), SC (3) | 0.9–16.0 |
| Total | 28:20 | 60.2 | 48 cases (50) | 0.7–16.0 |

CMSEP, colonic muco-submucosal elongated polyps; M, male; F, female; SC, sigmoid colon; SI, small intestines; DC, descending colon; AC, ascending colon; NS, not specified; TC, transverse colon; R, rectum.
prolapse polyps in patients with mucosal prolapse syndrome (MPS). MPS comprises a variety of clinico-pathologic entities with mucosal prolapse as the underlying pathogenic mechanism. Solitary rectal ulcer syndrome (SRUS) is the most common disorder of MPS. Gross and microscopic findings of SRUS show solitary or multiple ulcerated or polypoid lesions with crypt hyperplasia and villiform or serrated change and characteristic fibromuscular proliferation in the lamina propria. However, our case does not match SRUS clinically and pathologically. Another differential diagnosis to be considered is filiform polyposis which is characterized by numerous long slender “worm-like” projections in patients with inflammatory bowel disease. Filiform polyposis is generally thought to be a post-inflammatory reparative process of inflammatory bowel disease, and it is also referred to as inflammatory polyposis or pseudopolyposis. There have been reported cases of giant filiform polyposis associated with inflammatory bowel disease. Hamartomatous polyps are also included in the differential diagnosis due to the presence of proliferation of submucosal components such as blood vessels, hypertrophied muscularis mucosae, and mature adipose tissue. Peutz-Jegher polyps present hypertrophied branching frameworks of smooth muscles of the muscularis mucosae and the Cronkhite-Canada polyps reveal cystically dilated mucus glands with inflamed edematous stroma. However, there are no branching frameworks of muscularis mucosae and active inflammation in our case. The possibilities of ganglioneuroma, inflammatory myofibroblastic tumor, inflammatory fibroid polyp, and desmoid tumor could also be ruled out.

The exact pathogenesis of CMSEPs is currently unknown. Some suggest that CMSEPs show the clinico-pathologic features of mucosal prolapse polyps and the peristaltic movement of the gastrointestinal tract serves as a mechanical traction for redundant mucosa, in which such areas of the mucosa become the starting point of polyp formation. proposed that focal areas with prominent submucosal venous plexus may elevate the mucosa, thus becoming the leading point for the traction and ultimately leading to the polyp formation. presented a similar explanation for the formation of polypoid prolapsing mucosal folds in diverticular disease. However, we suggest that a combination of both mucosal prolapse and a hamartomatous process may produce a giant variant of CMSEP.

The CMSEP is a non-neoplastic polyp with a unique characteristic “worm-like” appearance. Due to the polyps being covered by normal mucosa and submucosal layers, at times, the superfi-

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

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Primary Follicular Lymphoma of the Duodenum: A Case Report

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Follicular lymphoma (FL) is a neoplasm of the germinal center B-cells and usually involves lymph nodes. Primary intestinal FL is an uncommon variant of FL. Among them, FL that involves the duodenum is reported separately as primary FL of the duodenum (PFL-D) for its unique indolent clinical course. PFL-D frequently involves the second portion of the duodenum, and it has been reported to have a very good prognosis even without any treatment. Due to its rarity and indolent behavior, unlike nodal FL, pathologists should be careful not to make under, or particularly, overdiagnosis. Recently, we experienced a case of PFL-D.

CASE REPORT

A 47-year-old male patient with no remarkable past medical history underwent gastroduodenoscopy as a part of preventive medical examination. Endoscopically, whitish multiple small mucosal nodules were noted adjacent to the major duodenal papilla. The lesion was confined to the mucosal layer (Fig. 1A, B). A biopsy was taken on suspicion of duodenal adenoma.

Microscopically, a polypoid lesion composed of a single prominent lymphoid follicle was demonstrated in the mucosa. The germinal center of the follicle mainly consisted of uniform centrocyte-like cells without tingible body macrophages. Small lymphoid cells similar to those found in the germinal center of the lesion were also present in the extrafollicular lamina propria of the villi (Fig. 1C). The submucosa was unremarkable. The tumor cells were immunopositive for CD20, CD10, Bcl-6, and Bcl-2. Ki-67 reactivity rate was very low (Fig. 1D, E). Notably, CD21-positive follicular dendritic cells (FDC) were condensed and redistributed at the periphery of the neoplastic follicle as described before (Fig. 1F).

The patient underwent staging work-up including laboratory tests, positron emission tomography computed tomography, and bone marrow biopsy. No evidence of systemic involvement was identified. In the process of retrospective review of the patient’s medical history, we found that the patient had annually undergone gastroduodenoscopy since 2012. We re-examined the biopsy slide that had been diagnosed as benign lymphoid follicular aggregation in the past, and confirmed that the lesion was also PFL-D by immunohistochemistry. There has been no endoscopic aggravation of the lesion during the past 2 years.

DISCUSSION

In this paper, we report a case of PFL-D that is a rare variant of FL. Schmatz et al. retrospectively reviewed 63 cases of PFL-D for a median follow-up time of longer than 6 years. In that study, PFL-D occurred once per 3,000 to 7,000 gastroduodenoscopies and showed good prognosis even in heterogeneous treatments including watch and wait, radiation, Rituximab monotherapy, and chemotherapy. Of note, seven cases showed complete regression without any treatment. However, a case of high grade transformation of PFL-D was reported recently.

Differentiation between PFL-D and reactive lymphoid hyperplasia or other B-cell lymphomas can be problematic. Normal follicles have tingible body macrophages in contrast to the neoplastic follicles in PFL-D. Centrocyte predominance lacking normal polarization is another characteristic of the neoplastic follicle. Strong immunopositivity for Bcl-2 and low immunopositivity

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for Ki-67 in the neoplastic follicles in addition to CD10 and Bcl-6–positive cells in extrafollicular region are also distinguishing features of PFL-D from benign follicular hyperplasia. Unlike nodal FL that shows high grade histology (grade 3) in 10% to 20% of the cases,3,4 our case also shows histologic features of the grade 1. Another notable feature is the pattern of FDC arrangement. Meshworks of FDC in nodal FL are present in follicular areas.5 However, a majority of PFL-D including our case show severely disrupted follicular pattern as in the follicular colonization of the MALT lymphoma. CD21 or CD23 immunohistochemical stain highlights rearrangement of FDC around the periphery of the neoplastic follicles.26 Takata et al.3 suggested that PFL-D be considered as a distinct entity based on this specific FDC pattern in addition to activation-induced cytidine deaminase loss and BACH2 expression.

In summary, in duodenal multi-nodular or multiple small polypoid lesions with prominent lymphoid follicles, PFL-D should be considered as one of the differential diagnoses. To our knowledge, the present case is the first reported case of PFL-D in Korea. Making an accurate diagnosis of PFL-D is important for the proper work-up in order to avoid an aggressive over-treatment.

Conflicts of Interest

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

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Patients with neurofibromatosis type 1 (NF-1) have rarely been reported to present with intraabdominal tumors including neuroendocrine tumors (NETs) and gastrointestinal stromal tumor (GIST), besides diagnostic features of café au lait macules, axillary or inguinal freckling, optic nerve gliomas, Lisch nodules, or neurofibromas. Specifically, the co-existence of periampullary NET and GIST has been suggested to be an indirect evidence for undisclosed NF-1. Herein, we report a NF-1 patient who presented with a combination of goblet cell carcinoid (GCC), NETs, and neurofibromatosis in the rectum, as well as a GIST in the jejunum.

CASE REPORT

A 46-year-old woman who congenitally had clinical features of NF-1 such as multiple neurofibromas of skin and café au lait macule has suffered from recurrent NETs in rectum since she received an operation for ovarian mucinous cystadenocarcinoma in 2010. The ovarian tumor showed neither recurrence nor distant metastasis during follow-up. She denied all the symptoms indicative of gastrointestinal diseases or carcinoid syndrome. Upon colonoscopy, the rectal mucosa revealed multifocal whitish patches, multiple biopsies from which revealed a coincidental occurrence of GCC, NETs, and neurofibromatosis. The abdominal computerized tomographic scan incidentally detected a submucosal mass in the jejunum. She underwent a low anterior resection of rectum and a wedge resection of jejunum. During the operation, the appendix was unremarkable by inspection. Grossly, the involved segment of rectum was about 10 cm in length and contained four to five patchy indurated lesions measuring 5.2 × 3.5 cm across in the largest one, which were limited to the mucosa and submucosa. Microscopically, the tumor consisted of small nests of signet-ring-like goblet cells (Fig. 1A, B), which positively stained with alcian blue pH 2.5 (Fig. 1C). Immunohistochemical stains for CD56, synaptophysin (Fig. 1D), and chromogranin A revealed scattered neuroendocrine cells, leading to the diagnosis of rectal GCC. Adjacent to rectal GCC, there were multifocal minute grade 1 NETs according to the grading system of 2010 World Health Organization (WHO) classification of NETs (Fig. 2A, B). Neurofibromatosis in the background of GCC and NETs was confirmed by S-100 protein immunostain (Fig. 2C, D). The submucosal tumor in the jejunum was composed of bland-looking spindle cells which expressed both c-kit and CD34 by immunohistochemistry, and diagnosed as very low risk GIST (size, 1.0 cm; mitotic count, < 1/50 high power fields) (Fig. 3A, B). Ten months after the operation, the patient was alive without recurrence or metastasis.

DISCUSSION

Even though GCC has been regarded as a mucin-producing NETs exclusively arising in the appendix, it is unsettled whether GCC should be classified as part of NETs or as variants of adenocarcinoma. The different histogenesis, the relative paucity of neuroendocrine cells, and more aggressive behavior have made GCC look different from NETs. It has been supposed that the dual neuroendocrine and intestinal differentiation in GCC emerge from undifferentiated stem cells at the base of the intestinal crypts,
whereas NETs initiate from subepithelial neuroendocrine cells in the mucosa. Moreover, the neuroendocrine cells of GCC are considerably fewer than those of NETs. As GCC has been more aggressive than NETs, adjuvant therapy after resection could be attempted commonly. Nevertheless, the genetic resemblance of GCC to NETs has precluded its being categorized as a subtype of adenocarcinoma. Indeed, GCC had allelic losses of 11q, 16q, or 18q which are frequent in gastrointestinal NETs, whereas KRAS, DPC4, or CTNNB1 mutations signaling for colorectal adenocarcinoma were undiscovered. The co-occurrence of GCC and NETs in this case might also implicate the close oncogenic relationship between them.

The rectum is an unusual site for GCC, and less than 10 cases of GCC arising in the rectum have been reported. Among them, there have been no patients with NF-1 as the present case, while a NF-1 patient with appendiceal GCC was recently described. Somatic inactivation of the NF1 has been the molecular hallmark of NF-1. A recent analysis has demonstrated that unlike sporadic GISTs, NF-1–related GISTs did not have activating mutations of KIT or PDGFRA, but showed somatic inactivation of NF1 more frequently. In addition, the tumor cells of gastric NET in a NF-1 patient showed germline nonsense mutation and loss of heterozygosity of NF1. Therefore, the occurrence of GCC along with NET, GIST and neurofibromatosis in this patient suggest that GCC might be one of NF-1–related tumors, and might have arisen from NF1 gene dysregulation. However, the genetic association between GCC and NF-1 needs to be established further.

Rectal GCC should be differentiated from metastasis of appendiceal GCC and from signet ring cell carcinoma, which usually forms luminal space and is negative for neuroendocrine markers by immunohistochemistry. Although the present patient had ovarian mucinous cystadenocarcinoma, the microscopic features between the tumors of rectum and ovary were obviously different.

In conclusion, for the first time we report rectal GCC arising in a NF-1 patient. This case will contribute to broadening the scope of gastrointestinal tumors, which NF-1 patients can harbor. More data need to be accumulated to determine the biologic characteristics and the optimal treatment strategies for this rare tumor.

Fig. 1. The rectum shows a combined occurrence of goblet cell carcinoid (GCC, empty arrows) and neuroendocrine tumors (filled arrows) as well as neurofibromatosis (A). GCC is mostly composed of small nests of signet-ring-like goblet cells (B), and the cytoplasmic mucin is readily identified with alcian blue pH 2.5 (C). (D) Synaptophysin immunostain clearly shows a few scattered neuroendocrine cells in GCC.
Conflicts of Interest

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

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Fig. 2. Neuroendocrine tumors (NETs) consist of small uniform cells which exclusively form trabecular structures (A) and diffusely stained with synaptophysin (B). In the vicinity of both goblet cell carcinoid and NETs, proliferation of slender spindle cells morphologically compatible with neurofibromatosis (C) is evident with S-100 protein immunostain (D).

Fig. 3. The submucosal tumor in the jejunum is composed of bland-looking spindle cells (A) which highly expressed c-Kit by immunohistochemistry (B).
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