

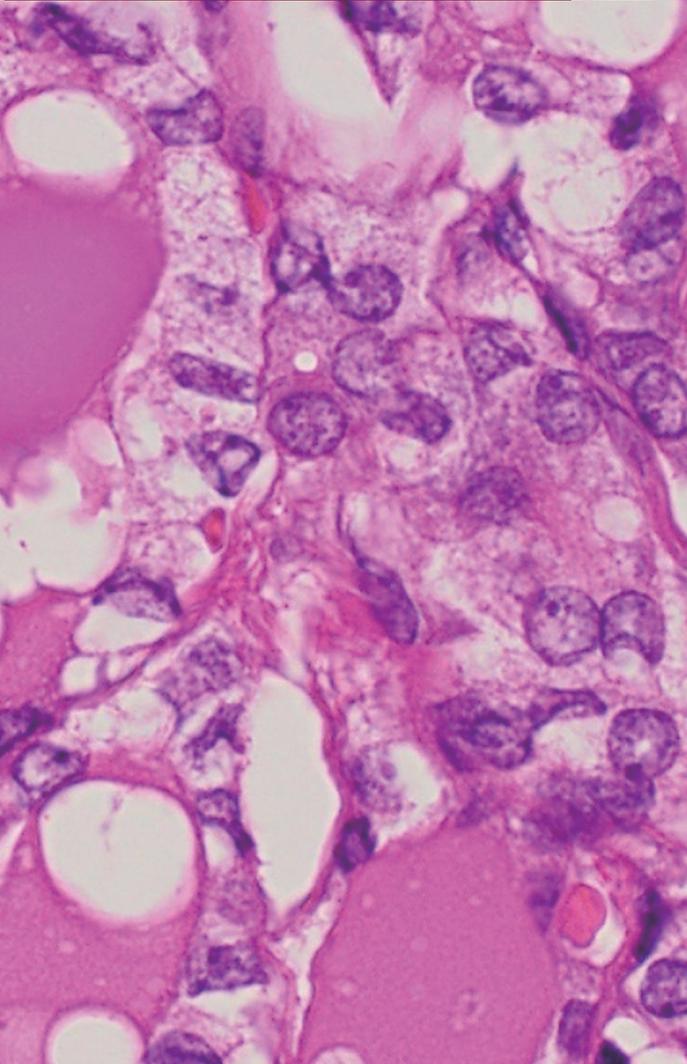
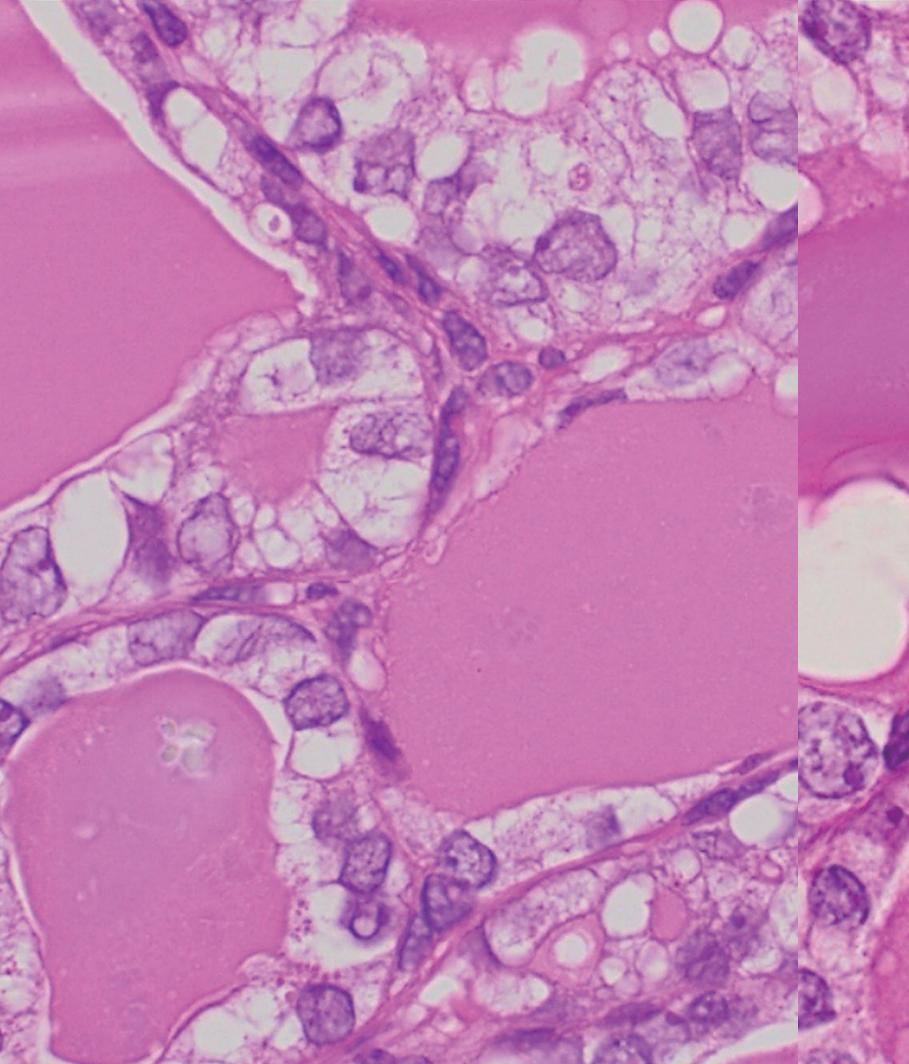
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*Nuclear Features of
Follicular Patterned
Thyroid Tumors*



Aims & Scope

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

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The Asian Thyroid Working Group, from 2017 to 2023

Kennichi Kakudo¹, Chan Kwon Jung², Zhiyan Liu³, Mitsuyoshi Hirokawa⁴, Andrey Bychkov⁵, Huy Gia Vuong⁶, Somboon Keelawat⁷, Radhika Srinivasan⁸, Jen-Fan Hang⁹, Chiung-Ru Lai⁹

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The Asian Thyroid Working Group was founded in 2017 at the 12th Asia Oceania Thyroid Association (AOTA) Congress in Busan, Korea. This group activity aims to characterize Asian thyroid nodule practice and establish strict diagnostic criteria for thyroid carcinomas, a reporting system for thyroid fine needle aspiration cytology without the aid of gene panel tests, and new clinical guidelines appropriate to conservative Asian thyroid nodule practice based on scientific evidence obtained from Asian patient cohorts. Asian thyroid nodule practice is usually designed for patient-centered clinical practice, which is based on the Hippocratic Oath, "First do not harm patients," and an oriental filial piety "Do not harm one's own body because it is a precious gift from parents," which is remote from defensive medical practice in the West where physicians, including pathologists, suffer from severe malpractice climate. Furthermore, Asian practice emphasizes the importance of resource management in navigating the overdiagnosis of low-risk thyroid carcinomas. This article summarizes the Asian Thyroid Working Group activities in the past 7 years, from 2017 to 2023, highlighting the diversity of thyroid nodule practice between Asia and the West and the background reasons why Asian clinicians and pathologists modified Western systems significantly.

Key Words: Asia; Thyroid neoplasms; Fine needle aspiration (FNA); Diagnostic criteria; Patient centered care; Active surveillance; Thyroidectomy

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While universally guided by fundamental principles, the discipline of pathology is inherently influenced by regional variations. Asian pathologists and cytopathologists, despite being educated predominantly through international textbooks written by Western scholars, often find that the ground realities and nuances in their regions necessitate adaptations. Systems like the Bethesda System for Reporting Thyroid Cytology (TBSRTC) [1], the World Health Organization (WHO) classification of thyroid tumors [2,3], and the American Thyroid Association clinical guidelines [4] are indispensable. Nevertheless, their direct application in Asia occasionally yields outcomes that diverge from Western findings [5-16]. The intersection of these

variances traces back to a myriad of intertwined scientific and non-scientific factors.

Population-based factors

Beyond genetic and biological differences, cultural practices and socio-environmental determinants in Asia can greatly influence thyroid pathology outcomes. For instance, dietary iodine intake, prevalent in many Asian diets, can significantly affect thyroid physiology and pathology.

Socio-economic and healthcare infrastructure

Diverse Asian nations, with their distinct historical trajectories,

have developed unique healthcare infrastructures. The accessibility and quality of healthcare, including diagnostic facilities, can vary even within countries, let alone between them.

Economic burden of medical care

The juxtaposition of North American and Asian healthcare expenditure strategies underlines more profound socio-economic and policy-driven differences. These variations often dictate clinical decisions, with patient affordability playing a crucial role. Medical expenses vary significantly across countries, with North America typically experiencing higher costs. Immediate surgery is frequently chosen to reduce these expenses. However, in other parts of the world, the approach often leans towards risk stratification for surgery and long-term clinical monitoring, as these tend to be more cost-effective than surgical interventions compared to the United States.

Healthcare insurance variations

Health insurance schemes' dynamics, influenced by public policies and private market forces, can significantly sway diagnostic and treatment choices.

Medical specialization and density of pathologists

The density of pathologists across countries significantly shapes the landscape of medical practice [17]. The number of pathologists in a country is intrinsically linked to the efficiency and effectiveness of its medical system.

Medico-legal climate and clinical guidelines

The medico-legal environment molds clinical practice considerably. In some countries, clinical practice guidelines are established with a defensive approach due to the potential for physicians to face malpractice lawsuits. While defensive medicine may seem prudent in litigious societies, it can inadvertently lead to overdiagnosis and overtreatment, escalating healthcare costs. Conversely, other nations resist such defensive medicine because it escalates societal costs and burdens patients financially [18-20].

Terminological and classificatory nuances

The evolution of medical terminologies and classifications, often reflecting deeper understandings of diseases, underscores the importance of constant knowledge exchange and adaptation among global medical communities. The noninvasive encapsulated follicular variant of papillary thyroid carcinoma (FVPTC) was once classified as a malignant tumor in American thyroid nodule practices. Its prevalence in the West is elevated due to a

lower threshold for recognizing papillary thyroid carcinoma (PTC) nuclear features (Fig. 1) [21,22]. However, this classification was later changed to noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) [2,23]. In many Asian practices, these are typically observed in benign follicular adenomas (FAs) due to a higher threshold for PTC-type nuclear features (Fig. 1), resulting in a lower prevalence of NIFTP in surgically removed nodules in Asia [2,8,11,12,14-16].

Clinical management philosophies

The decision-making algorithms in medicine are based on clinical evidence, patient preferences, physician experiences, and systemic constraints. In Asian clinical practice, a more conservative management approach is typically adopted, emphasizing clinical risk stratification for high-risk indeterminate thyroid nodules, specifically follicular neoplasm (FN) [9-11,13-16]. Conversely, in North America, diagnostic surgery becomes the preferred course when patients cannot afford molecular tests [1,4,9,10,11,13-15]. Consequently, in the West, indeterminate nodules, categorized as Bethesda III (atypia of undetermined significance [AUS]) and IV (FN), have a high resection rate (RR) of over 50% but a lower risk of malignancy (ROM) of less than 30%. In contrast, most Asian practices show a lower RR of less than 40% for these nodules, with a ROM exceeding 40%, even in the absence of molecular testing [9-11,13-16].

Country-specific approaches

Each country, influenced by its unique medical history, patient demographics, and healthcare policies, carves out its distinct approach to disease management. In Japan, a more conservative approach to management is prevalent [10,11,24-26]. Traditionally, lobectomy is the chosen procedure for low-risk small PTCs measuring 1–2 cm and for slightly larger low-risk PTCs, specifically, those sized less than 4 cm and categorized as T1–2, extrathyroidal extension (Ex) 0–1, N0–1, and M0 [24-26]. This conservative strategy offers notable benefits, most crucially the potential preservation of thyroid function. Such a conservative approach is particularly vital as patients who undergo total thyroidectomy and subsequently experience poorly managed hypothyroidism face severe risks, especially as they age and potentially struggle with proper hormone supplement management. Contrarily, in most other regions of Asia, as well as in North America and Europe, the predominant choice leans towards total thyroidectomy in the past [4,24-27].

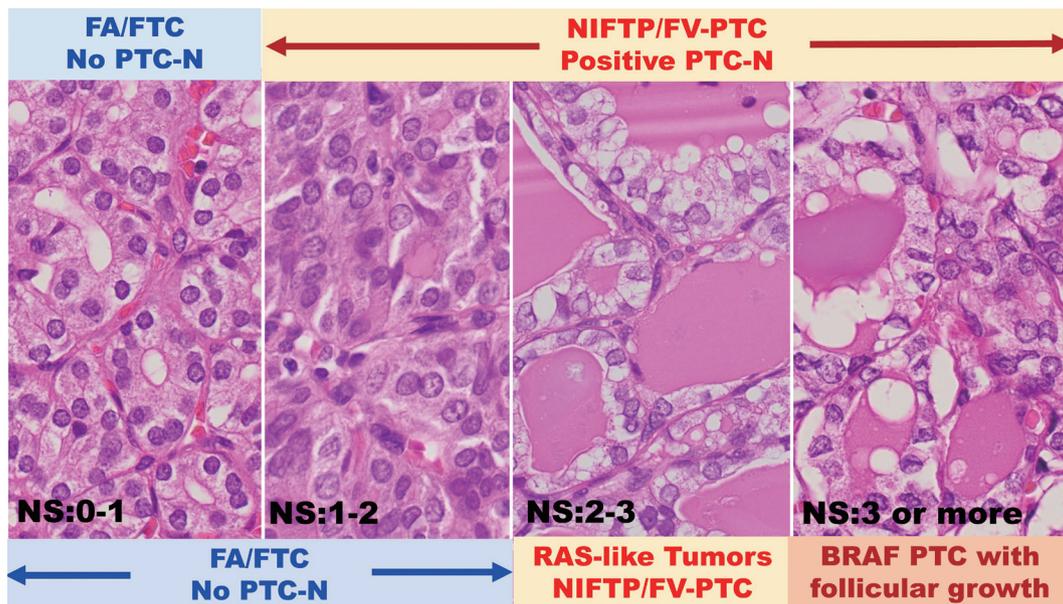


Fig. 1. NS from 0 to 3 in encapsulated follicular pattern tumors results in variable benign, borderline, or malignant diagnoses between Asian and Western pathologists. Four illustrations (NS 0–1, NS 1–2, NS 2–3, and NS 3 or more) in encapsulated follicular pattern tumors often result in diverse benign, borderline, or malignant diagnoses among pathologists [5–7]. The two illustrations on the left are FA when noninvasive or FTC when invasive in Asian thyroid practice because those extremely delicate nuclear features are insufficient for PTC-type malignancy. Most Western pathologists accept nuclear features in the three illustrations (NS 1–2, NS 2–3, and NS 3 or more) on the right as positive for PTC-N and call them using the same diagnostic terminology, PTC type nuclear features, regardless of different genetic backgrounds, *BRAF* or *RAS* oncogene lineages [1–3,21–23]. Some Asian pathologists distinguish nuclear features in the two illustrations (NS of 2–3 and NS of 3 or more) on the right, either *RAS*-like FV-PTC or *BRAF*-like conventional PTC [14,16,35]. NS, nuclear score defined by Nikiforov et al. [2,23]. NS, nuclear scoring; FA, follicular adenoma; FTC, follicular thyroid carcinoma; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; PTC, papillary thyroid carcinoma; PTC-N, papillary thyroid carcinoma type nuclear features; FV-PTC, follicular variant PTC; *RAS*, rat sarcoma virus; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B.

Active surveillance vs. surgical intervention

The balance between surgical intervention and observation hinges on the collective experiences of a medical community, patient trust, and the broader healthcare framework. In Japan, over half of the patients with low-risk, small PTCs (less than 10 mm) opt for active surveillance, undergoing clinical follow-ups without surgery once a fine needle aspiration (FNA) confirms the malignancy [4,28–31]. Conversely, in the majority of other nations, FNA is typically not recommended for thyroid nodules that are small and low-risk, especially those under 1 cm [4]. This distinction arises from the fact that once an FNA identifies a nodule as malignant, surgery becomes the frequent choice, leading to potential overtreatment for patients with low-risk, small PTCs [4,14–16].

Grossing techniques for encapsulated follicular pattern thyroid tumors

As medicine advances, so does the need for precision. Nevertheless, precision must be juxtaposed against practicality in the

light of resource optimization. Grossing techniques for encapsulated follicular pattern thyroid tumors vary globally. In Western countries, where pathologists often adopt defensive medical practices due to malpractice concerns, there is an emphasis on meticulously sampling the entire tumor capsules [2,15,16,32,33]. As recommended in Western textbooks, this practice has influenced the protocols in certain Asian laboratories, notably in China, Korea, Taiwan, and Thailand [32]. Conversely, Indian, Indonesian and Japanese labs generally select only representative sections, typically fewer than 10 per nodule, adhering to prior textbook guidelines [33,34]. This streamlined approach reduces the number of hematoxylin and eosin sections taken (from more than 20 sections to fewer than 10 per nodule) and conserves both pathologist time and societal resources.

The reasoning behind this more economical approach in India, Indonesia and Japan is multifaceted: (1) Lobectomy is deemed suitable for a range of nodules, from benign (e.g., FA) to borderline tumors (e.g., NIFTP, thyroid tumors of uncertain malignant potential), and even low-risk malignancies (like minimally

invasive follicular thyroid carcinoma [FTC] or encapsulated conventional PTC) [4,24-26,33]. (2) Only pronounced, clear invasions carry significant implications for a patient's prognosis. With this method focused on Sustainable Development Goals, there is virtually no risk of overlooking clinically consequential carcinomas, such as extensively angio-invasive or widely invasive carcinomas [2,3,33]. (3) Conducting exhaustive histological examinations to identify ambiguous invasions can result in the overtreatment of low-risk tumors, such as those termed minimally invasive FTCs or encapsulated FVPTCs (EFVPTCs). (4) Restricting the number of samples (to less than 10 sections) mirrors the clinical guideline of refraining from FNAs on low-risk thyroid nodules smaller than 1 cm. This approach encourages a clinical follow-up, subsequently reducing surgeries for small papillary carcinomas. Moreover, limiting samples can mitigate the unintended identification of questionable capsular invasions and, consequently, the diagnoses of minimally invasive FTCs and EFVPTCs. This, in turn, lessens the likelihood of excessive treatments, like completion thyroidectomy or radioactive iodine ablation, particularly for very low-risk thyroid tumors with negligible prognostic impact [36-39].

Categorization of cyst fluid only samples

How we classify and interpret diagnostic samples evolves with our understanding of disease processes and the nuances of clinical implications. In past cytological practices, including in the United States, cyst fluid only samples from thyroid FNA cytology were typically categorized as benign. However, current Western practices have moved these samples to the inadequate category due to the substantial risk they pose for false-negative diagnoses and the potential inability to completely exclude cystic PTC [1,14-16]. In contrast, some countries in Asia/Oceania still classify these samples as benign, while European countries might place them in a specific subcategory under the inadequate category. This reclassification in Europe is attributed to the extremely low malignancy risk associated with these samples, which is on par with or even lower than the risk associated with samples in the benign category [9,15,16].

VARIATION IN THYROID NODULE PRACTICES ACROSS REGIONS

In 2002, some members of the Asian Thyroid Working Group observed notable discrepancies between Japanese and American pathologists concerning the classification of encapsulated follicular pattern thyroid tumors [5,6]. The majority of noninvasive

encapsulated follicular pattern thyroid nodules exhibiting subtle nuclear atypia consistent with RAS-like tumors (RAS-like dysplasia) were classified as malignant PTCs by American pathologists. In contrast, Japanese pathologists often categorized them as benign FAs or hyperplastic nodules (Fig. 1) [5,6,16,21,22]. This observation was further corroborated by multiple subsequent studies [7,15,21,22]. Following a comprehensive review of 109 cases, which exhibited neither recurrence nor metastasis over an average 14-year follow-up period, the international NIFTP working group reclassified these noninvasive encapsulated follicular pattern tumors in 2016. Previously deemed as malignant (FVPTC), they were redefined as borderline tumors (NIFTP) [23]. Despite this, considerable disparities persist between Asian and Western practices, notably in the prevalence of NIFTP—a statistic that remains higher in the West [2,8,11,12,14-16,22,40-43] compared to Asia [12,14,44-49].

The Asian Thyroid Working Group was inaugurated in 2017 during the 12th Asia Oceania Thyroid Association (AOTA) Congress in Busan, Korea [49,50]. The group's initial endeavors explored the distinctions between Asian and Western approaches to thyroid nodule management. In the West, FVPTC (often synonymous with RAS-like PTC) represents a significant portion of PTC diagnoses [1-4,12,22,23,37,51]. Consequently, the frequency of the *BRAF* V600E mutation is somewhat diminished in Western PTC cases, given that EFVPTCs are typically non-*BRAF* (RAS-like) tumors [40]. Conversely, Asian PTC samples manifest a higher prevalence of the *BRAF* V600E mutation, largely because EFVPTCs are scarcely identified in Asian thyroid evaluations. A majority of encapsulated follicular pattern tumors with mild RAS-like dysplastic nuclear alterations are designated as benign FA in prevalent Asian practices [6-8,11,14-16,40,41].

This manuscript chronicles the endeavors and discoveries of the Asian Thyroid Working Group from 2017 to 2023, underscoring the nuanced distinctions in thyroid nodule practices between Asia and the West. A comprehensive exploration of these global thyroid nodule practice variances will be the centerpiece of an upcoming textbook titled "Thyroid FNA Cytology, Differential Diagnoses, and Pitfalls: 3rd Edition" [52].

GROUP STUDY INSIGHTS

Asian perspectives on NIFTP [12,44-48]

A noninvasive encapsulated follicular pattern tumor, which exhibited worrisome nuclear features characteristic of PTC (RAS-like dysplasia), was previously categorized as a malignant tumor (FVPTC). This classification was predominant due to the lower

threshold set for malignancy based on RAS-like PTC nuclear features in earlier Western thyroid nodule practices [5-8,21-23, 40-45]. This classification was later revised to “NIFTP” and was categorized as a borderline tumor in the 4th edition of the WHO classification [2,23,42,43]. Comprehensive data regarding the global incidence of NIFTP was derived from a thorough review of published studies and collaborative research efforts by members of the Asian Thyroid Working Group. These studies ascertained that the global occurrence of NIFTP is notably less than what was initially anticipated [12,47,48]. Furthermore, the incidence rates of NIFTP in Asia are substantially lower than those observed in North American and European regions. This discrepancy stems from the perception of NIFTP in Asia—it isn’t deemed to necessitate surgical intervention. Instead, a conservative approach that emphasizes risk stratification for surgical interventions is the conventional clinical protocol for managing indeterminate thyroid nodules in Asia [9-11,14-16]. This practice is also influenced by the stringent criteria for identifying PTC-type nuclear features in the region (Fig. 1) [5,6,11,14-16,21,22,40].

Malignant lymphoma in Asian practice [53]

This study analyzed 153 cases of primary thyroid lymphoma (PTL) collected from 10 institutions represented by members of the Asian Thyroid Working Group. The reported prevalence was 0.54% of all malignant thyroid tumors. The breakdown indicated that mucosa-associated lymphoid tissue lymphoma accounted for 54.9%, while diffuse large B-cell lymphoma represented 38.6%. Ultrasound examination and FNA cytology were the primary preoperative diagnostic tools, with flow cytometry conducted in five institutions. The findings suggest that the prevalence of PTL in non-Western countries is lower than previously reported in other studies.

Oncocytic (Hürthle cell) lesions in Asian practice [54]

Of 42,190 thyroid aspirates, 760 (1.8%) exhibited a predominance of Hürthle cells. These samples were sourced from nine hospitals across six Asian countries. The majority, or 61%, were categorized as “atypia of undetermined significance, Hürthle cell type” (AUS-H); 35% were identified as “follicular neoplasm, Hürthle cell type” (FN-H); and 4% were classified as “suspicious for malignancy” (SFM). Histologic follow-up was conducted for 288 of these aspirates (equivalent to 38%). Of these, a significant majority (66%) were determined to be benign upon resection, with the most common histologic diagnosis being Hürthle cell adenoma at 28.5%. The ROM for AUS-H, FN-H, and SFM, based on the resected nodules, was 32%, 31%, and 71%, respec-

tively. Meanwhile, the risk of neoplasm was calculated to be 47%, 81%, and 77% for the respective categories.

Medullary (C cell) thyroid carcinoma in Asia practice [55-57]

From 13 hospitals across 8 Asia-Pacific countries, 145 cases of medullary (C cell) thyroid carcinoma (MTC) with accessible FNA slides were gathered for this study. Of these cases, 99 (68.3%) were preliminarily interpreted as either suspicious for MTC (S-MTC) or confirmed as MTC. While cytological detection alone for MTC showed limitations, the combined application with auxiliary tests substantially enhanced diagnostic proficiency. The staining techniques employed varied across institutions and included Papanicolaou, hematoxylin-eosin, and Romanowsky methods. Liquid-based cytology was implemented in merely three of the countries. Following a comprehensive review of all cases, the diagnostic rate for MTC or S-MTC rose to 91.7% (133 out of the 145 cases). Based solely on cytomorphologic data, a plausible scoring system has been suggested to ensure optimal diagnostic precision. Additionally, Jung et al. [57] provided an overview of the latest advancements and modifications related to MTC as delineated in the WHO classification.

Capsular invasion study [58]

This investigation assessed interobserver concordance in evaluating capsular invasion among 11 thyroid pathologists from five Asian countries. This was accomplished using 20 cases presented as virtual slides. The levels of agreement for definitive invasive and noninvasive classifications were fair, evidenced by kappa values of 0.578 and 0.404, respectively. However, concordance was poor for cases with ambiguous invasion, as indicated by a kappa value of 0.186. The discrepancies in invasion assessment led to divergent final pathological conclusions. In summary, the research highlighted significant interobserver variability in the assessment of capsular invasion, particularly in FNs where the invasion was debatable.

Anaplastic thyroid carcinoma studies [59,60]

PAX8 in anaplastic thyroid carcinoma [59]

PAX8 immunohistochemistry using the MRQ-50 antibody was performed in whole tissue slides (n = 147) or tissue microarray sections (n = 35). The study found PAX8 expression in 54.4% of the cases, significantly lower than those reported in prior studies with the polyclonal antibody. PAX8 expression was positively correlated with an epithelial pattern (63.6% vs. 37.5%) and a coexisting differentiated thyroid carcinoma component (71.6%

vs. 44.3%). Pathologists should be aware that PAX8 expression in anaplastic thyroid carcinoma (ATC) is less than those reported in early studies to avoid misdiagnosis.

Primary and secondary ATCs [60]

This study searched for ATCs in our institutional databases and the Surveillance, Epidemiology, and End Result (SEER) database. The multi-institutional database retrieved 22 primary (de novo) and 23 secondary ATCs (the patient had a history of differentiated thyroid cancer [DTC] or coexisting DTC components at the time of diagnosis). Compared to primary ATCs, secondary ATCs were not statistically different regarding demographics, clinical manifestations, and patient survival. The only clinical discrepancy between the two groups was a significantly larger tumor diameter of the primary ATCs. The prevalence of *TERT* promoter, *PIK3CA*, and *TP53* mutations was comparable between the two subtypes. In comparison to primary ATCs, however, *BRAF* mutations were more prevalent (odds ratio [OR], 4.70; 95% confidence interval [CI], 2.84 to 7.78), whereas *RAS* mutations were less frequent (OR, 0.43; 95% CI, 0.21 to 0.85) in secondary tumors.

BRAF-like nuclear features and RAS-like dysplasia [35]

This study examined whether pathologists could distinguish *BRAF*-like and *RAS*-like nuclear features morphologically. This analysis suggests that nuclear pseudo-inclusions and high nuclear scores have diagnostic utility as rule-in markers for differentiating PTC with *BRAF* V600E mutation from benign or borderline follicular tumors with *RAS*-like mutations. Relaxation of rigid criteria for nuclear features resulted in an overdiagnosis of PTC. Immunostaining or molecular testing for *BRAF* V600E mutation is a valuable adjunct for cases with high nuclear scores to identify true PTC.

FA with papillary architecture and a proposal for a new borderline tumor, noninvasive encapsulated papillary RAS-like thyroid tumor [61,62]

The term “noninvasive encapsulated papillary RAS-like thyroid tumor (NEPRAS)” was introduced by Ohba et al. in 2019 [61] to describe a noninvasive thyroid tumor characterized by a complete fibrous capsule, a predominantly papillary architecture, and the presence of a *RAS* mutation, yet exhibiting only subtle nuclear features consistent with PTC (RAS-like dysplasia). This tumor poses a challenge for pathologists as it lies at the intersection between an encapsulated conventional *BRAF*-like PTC and the FA with papillary architecture, which was recognized

as a benign tumor entity in the 5th edition of the WHO classification [2,3].

To address the diagnostic challenge and reduce the psychological impact on patients, the term “NEPRAS” was proposed, echoing the approach taken with the NIFTP terminology as introduced by Nikiforov et al. [23]. Subsequently, Jung et al. [62] documented three additional cases, suggesting that a favorable prognosis could be anticipated following surgical resection of such tumors. This optimism is grounded in the understanding that most encapsulated thyroid tumors, when not invasive, tend to be indolent, mirroring the behavior of FAs regardless of their growth patterns (be it NIFTP in follicular or NEPRAS in papillary patterns) [37,38,63]. However, it is noteworthy that extensive long-term follow-up data on a large patient cohort still needs to be available [62].

RR and ROM of indeterminate (Bethesda III and IV) cytology [13,64-66]

Without molecular tests [13,64]

Increasing evidence shows that clinicians employ different management strategies in their use of TBSRTC. This meta-analysis investigated the differences in diagnosis frequency, RR, and ROM between Western and Asian cytopathology practices. This study demonstrates a difference in Western and Asian thyroid cytology practice, especially regarding the indeterminate categories. Lower RR (51.3% vs. 37.6%) and higher ROM (25.4% vs. 41.9%) suggest that Asian clinicians adopt a more conservative approach, whereas immediate diagnostic surgery is favored in Western practice for indeterminate nodules.

With gene panel tests [65]

Compared with Afirma microarray-based Gene Expression Classifier, Gene Sequencing Classifier (GSC) had a higher benign call rate (BCR) (65.3% vs. 43.8%), a lower RR (26.8% vs. 50.1%), and a higher ROM (60.1% vs. 37.6%). The BCR of Hürthle cell-predominant nodules was significantly elevated (73.7% vs. 21.4%). In addition, the specificity (43.0% vs. 25.1%) and positive predictive value (63.1% vs. 41.6%) of Afirma GSC were significantly improved while it still maintained a high sensitivity (94.3%) and a high negative predictive value (90.0%). With an increased BCR and improved diagnostic performance, GSC could reduce the rate of unnecessary surgical interventions and better tailor the clinical decisions of patients with indeterminate thyroid FNA results.

With molecular tests in Asia [66]

This meta-analysis study included a total of 34 studies with 7,976 indeterminate nodules. The multigene panel testing methods were exclusively used in the United States. Compared with the non-molecular era, molecular testing was associated with a significantly increased ROM (47.9% vs. 32.1%). The ROM of indeterminate nodules in Asian institutes was significantly higher than in Western countries (75.3% vs. 36.6%). Institutes employing single-gene tests achieved a higher ROM (59.8% vs. 37.9%). Molecular testing is a promising method to tailor the clinical management for indeterminate thyroid FNA. The combination of molecular testing and active surveillance enhances the accuracy of case selection for surgery in Asian countries.

Pediatric thyroid carcinoma [67-69]

TBSRTC outputs, including frequency and ROM for most categories, were not statistically different from data in adult patients. However, the RR in the pediatric group was significantly higher in most of the categories compared with published adult data: benign, 23.2% vs. 13.0%; AUS, 62.6% vs. 36.2%; FN, 84.3% vs. 60.5%; and SFM, 93.8% vs. 69.7%. Pediatric patients with benign and indeterminate thyroid nodules had a higher RR than their adult counterparts, but the ROM of these categories in adults and children was not statistically different, suggesting a potential risk of overtreatment in pediatric patients. Determining the best treatment guidelines and additional tools for risk stratification must be a top priority to identify the target patient groups for surgical intervention precisely. Our study further demonstrated that Asian pediatric thyroid nodules had higher ROM than those from adults.

Coronavirus disease 2019 pandemic in Asian cytology practice [70]

This study examined the impact of coronavirus disease 2019 (COVID-19) on cytology practice in the Asia-Pacific region involving 167 cytopathology laboratories from 24 countries. The majority reported that restrictive measures that limited the accessibility of health care services had been implemented in their cities and/or countries (80.8%) and their hospitals (83.8%). Approximately one-half of the participants reported the implementation of new biosafety protocols (54.5%) and improving laboratory facilities (47.3%). The majority of the respondents reported a significant reduction (> 10%) in caseload associated with both gynecological (82.0%) and nongynecological specimens (78.4%). Ten out of 14 authors were from the Asian Thyroid Working Group members.

Other meta-analyses of the literature [71-74]

Major fusion oncogenes in PTC [71]

This meta-analysis using 27 studies showed *NTRK*-, *RET*-, *BRAF*-, and *ALK*-rearranged PTCs had a unique demographic/clinicopathological profile but similar progression-free survival (PFS) and overall survival. *NTRK1*-positive PTCs demonstrated more aggressive clinical behaviors and shorter PFS than *NTRK3*-positive PTCs, whereas *RET* rearrangement variants shared comparable clinicopathological backgrounds. This study provides new insights and facilitates our understanding of clinicopathological features and survival outcomes of different fusion oncogenes in PTCs.

The metastatic pattern of thyroid carcinomas [72]

We included 2,787 M1 thyroid cancers for statistical analyses, and the incidence of distant metastasis at presentation was 2.4%. Lung was the most common metastatic site for ATC, poorly differentiated thyroid carcinoma, PTC, and oncocytic cell carcinoma, whereas bone is the favorable disseminated site of FTC and MTC. Patients with multi-organ metastases had the worst survival, whereas bone metastases were associated with a favorable outcome. Significant differences exist in distant metastasis patterns of thyroid cancer subtypes and their corresponding survival.

Malignant thyroid teratoma [73]

We incorporated the SEER data with published malignant thyroid teratoma (MTT) cases in the literature to analyze the characteristics and prognostic factors of MTTs. Our results showed that MTT is typically seen in adult females. These neoplasms were associated with an aggressive clinical course with high rates of extrathyroidal extension (80%) and nodal involvement (62%). During follow-up, the development of recurrence and metastases were common (42% and 46%, respectively), and one-third of patients died at the last follow-up.

Mucoepidermoid carcinoma and sclerosing mucoepidermoid carcinoma with eosinophilia [74]

This multicenter study of mucoepidermoid carcinoma (MEC) and MEC with eosinophilia (SMECE) integrated our data with published literature to further investigate these tumors' clinicopathological characteristics and prognoses. Histopathologically, MECs and SMECEs comprised two main cell types, including epidermoid and mucin-secreting cells, arranged in cords, nests, and tubules. SMECEs were characterized by a densely sclerotic stroma with abundant eosinophils. SMECEs had a superior dis-

ease-specific survival rate compared to MECs, suggesting that they are low-grade cancers. This could help clinicians better evaluate patient outcomes and decide appropriate treatment plans.

Future group studies in the Asian Thyroid Working Group

One of the most promising and valuable projects for pathologists is revising thyroid tumor classification, such as refining new tumor entities and establishing a molecular classification of thyroid tumors. The current WHO classification is insufficient for genuine molecular classification, and Western pathologists distinguish RAS-mutated encapsulated follicular pattern thyroid tumors into two tumor groups, FA/tumors of uncertain malignant potential (UMP)/FTC and NIFTP/UMP/FVPTC, according to the absence (NS 0–1) or presence (NS 2–3) of RAS-like dysplastic nuclear features (Fig. 1). This distinction has very little clinical imprecision because both are treated similarly, and the outcomes are almost identical [2,4]. In Asian thyroid practice, the distinction between FA/UMP/FTC and NIFTP/UMP/FVPTC is not strict and often handles them into one tumor lineage, FA/FTC. Thus, NIFTP and FVPTC are rare in Asia [12], while FTC is a vanishing tumor entity and is frequently classified as FVPTC in recent Western practice [75,76]. The lack of uniformity in diagnosing encapsulated follicular pattern tumors with delicate RAS-nuclear features among pathologists creates serious confusion in referring clinicians in some instances and hinders data sharing among institutes. It is a time to combine both RAS-like tumors into one tumor entity, as there are no benefits in the strict sub-classification of RAS-like tumors (FA/UMP/FTC vs. NIFTP/UMP/FVPTC) for treating physicians and patients. The author believes that universally accepted handling of the same RAS oncogene-mutated thyroid tumors is essential to minimize observer variation of encapsulated follicular pattern tumors and establish better communication between Asia and the West, harmonizing them into one world.

SPECIAL ISSUES CONDUCTED BY THE ASIAN THYROID WORKING GROUP MEMBERS

Since 2016, multiple Asian Thyroid Working Group members have curated special issues in various journals. These special issues include:

Journal of Basic & Clinical Medicine

In 2016, a pivotal moment in pathology occurred with Niki-forov's publication of a seminal paper introducing the borderline tumor entity known as NIFTP [23]. Recognizing the im-

portance and potential implications of this new classification, Dr. Kakudo, in the same year, invited a distinguished cohort of 15 international authors, inclusive of four members from the Asian Thyroid Working Group, to present their perspectives and insights on NIFTP in the *Journal of Basic & Clinical Medicine* (Table 1). It is unfortunate that the journal above subsequently ceased its operations, and its digital presence has vanished. Nevertheless, most of the seminal works, including those discussing NIFTP, are retrievable online, specifically at Dr. Kakudo's website (http://www.kakudok.jp/english/basic_and_clinical_medicine/) and the individual ResearchGate profiles of the respective authors.

Journal of Pathology and Translational Medicine

In the AOTA Busan meeting, the Asian Thyroid Working Group commenced its operations in 2017. To begin their collaborative efforts, the Asian Thyroid Working Group summarized and reported the prevailing status of thyroid FNA cytology practices across seven Asian nations. These findings were subsequently published in a special issue of the *Journal of Pathology and Translational Medicine*, sponsored by the Korean Society of Pathologists and the Korean Society for Cytopathology. The articles from this special issue are delineated below in Table 2.

Gland Surgery

"Asian and Western Practice in Thyroid Pathology: Similarities and Differences" was a themed issue published in *Gland Surgery* (Table 3). It addressed critical differences observed when Western systems for thyroid pathology and cytology were adopted in Asian practice. Dr. Kakudo invited 25 international authors, of which 19 are members of the Asian Thyroid Working Group. These original articles, reviews, and meta-analyses highlight significant differences. Some of these disparities are entirely understandable and expected, while others are deemed scientifically unacceptable and not suitable for patient-centered care.

THE ASIAN THYROID WORKING GROUP COMPANION MEETINGS

The inaugural Asian Thyroid Working Group Companion Meeting was convened at the 18th AOTA Congress in Busan, Korea, on March 16, 2017. Subsequent Companion Meetings have been held annually, as detailed in Tables 4–8. However, the meetings scheduled for 2020 and 2021 were canceled due to the COVID-19 pandemic.

Table 1. Publications on NIFTP in the *Journal of Basic & Clinical Medicine*, 2017

| No. | Author(s) | Title | Volume, page |
|-----|---|---|--------------|
| 1 | Kakudo K [77] | Non-invasive encapsulated/well-circumscribed follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and precursor thyroid tumors | 6(1):1-2 |
| 2 | Kakudo K [78] | Unsettled issues in non-invasive encapsulated/well-circumscribed follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and precursor thyroid tumors | 6(1):3-7 |
| 3 | LiVolsi VA, Baloch ZW [79] | Coming to terms with diagnosis "Non-invasive follicular neoplasm with papillary-like nuclear features (NIFTP)": practice changer in endocrine pathology | 6(1):8-13 |
| 4 | Kakudo K, Liu Z, Satoh S, Higuchi M, Hirokawa M [80] | Non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP): diagnosis and differential diagnoses | 6(1):14-21 |
| 5 | Liu Z, Song Y, Han B, Zhang X, Su P, Cui X [81] | Non-invasive follicular thyroid neoplasm with papillary-like nuclear features and the practice in Qilu Hospital of Shandong University, China | 6(1):22-25 |
| 6 | Jung CK, Kim C [82] | Effect of lowering the diagnostic threshold for encapsulated follicular variant of papillary thyroid carcinoma on the prevalence of non-invasive follicular thyroid neoplasm with papillary-like nuclear features: a single-institution experience in Korea | 6(1):26-28 |
| 7 | Pusztaszeri MP, Triponez F, Meyer P, Sadowski SM [83] | Noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP): report of an institutional experience with 86 cases | 6(1):29-35 |
| 8 | Rossi ED [84] | NIFTP diagnosis: roses and thorns for cytopathologists and histopathologists | 6(1):36-37 |
| 9 | Canberk S, Baloch ZW, Ince U, Schmitt F [85] | Diagnosis of non-invasive follicular tumor with papillary-like nuclear features (NIFTP): a practice changer for thyroid fine-needle aspiration interpretation | 6(1):38-43 |
| 10 | Maletta F, Volante M, Papotti M [86] | Experience on NIFTP cytology, with a mini meta-analysis of the literature | 6(1):44-50 |
| 11 | Ng D, Can NT, Ma ZV, van Zante A, Ljung BM, Khanafshar E [87] | Cytomorphologic features of noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP): a comparison with infiltrative follicular variant of papillary thyroid carcinoma | 6(1):51-56 |
| 12 | Saglietti C, Bongiovanni M [88] | The value of cytological examination in the diagnosis of noninvasive thyroid neoplasm with papillary-like nuclear features (NIFTP) | 6(1):57-60 |
| 13 | Wu HH [89] | The impact of NIFTP on FNA cytology: can we still diagnose papillary thyroid carcinoma? | 6(1):61-62 |
| 14 | Yang GCH [90] | Ultrasound is cytopathologist's best friend in the era of noninvasive follicular thyroid neoplasm with papillary-like nuclear features | 6(1):65-67 |
| 15 | Poller DN, Johnson SJ, Stephenson TJ [91] | Diagnosis of NIFTP in the UK | 6(1):63-64 |

NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

Table 2. Articles on thyroid fine needle aspiration practices in Asia from *Journal of Pathology and Translational Medicine*, 2017

| No. | Author(s) | Title | Volume, page |
|-----|---|---|---------------|
| 1 | Bychkov A, Kakudo K, Hong S [49] | Current practices of thyroid fine-needle aspiration in Asia: a missing voice | 51(6):517-520 |
| 2 | Cha YJ, Pyo JY, Hong SW, et al. [92] | Thyroid fine-needle aspiration cytology practice in Korea | 51(6):521-527 |
| 3 | Liu Z, Dongge D, Ma B, et al. [93] | History and practice of thyroid fine-needle aspiration in China, based on retrospective study of the practice in Shandong University Qilu Hospital | 51(6):528-532 |
| 4 | Agarwal S, Jain D [94] | Thyroid cytology in India: contemporary review and meta-analysis | 51(6):533-547 |
| 5 | Satoh S, Yamashita H, Kakudo K [95] | Thyroid cytology: the Japanese system and experience at Yamashita Thyroid Hospital | 51(6):548-554 |
| 6 | Abelardo AD [96] | Thyroid fine-needle aspiration practice in the Philippines | 51(6):555-559 |
| 7 | Hang JF, Hsu CY, Lai CR [97] | Thyroid fine-needle aspiration in Taiwan: the history and current practice | 51(6):560-564 |
| 8 | Keelawat S, Rangdaeng S, Koonmee S, Jitpasutham T, Bychkov A [98] | Current status of thyroid fine-needle aspiration practice in Thailand | 51(6):565-570 |
| 9 | Jung CK, Hong S, Bychkov A, Kakudo K [50] | The use of fine-needle aspiration (FNA) cytology in patients with thyroid nodules in Asia: a brief overview of studies from the Working Group of Asian Thyroid FNA Cytology | 51(6):571-578 |

FUTURE PERSPECTIVES

Kennichi Kakudo served as the esteemed president of the Asian Thyroid Working Group from 2017 to 2023, concluding his tenure in July 2023. During this period, the core members who played a pivotal role alongside Dr. Kakudo were Chan Kwon Jung from Korea, Zhiyan Liu from China, Mitsuyoshi Hirokawa from Japan, and Andrey Bychkov, who had affiliations with Russia, Thailand, and Japan. In a significant transition,

Chiung-Ru Lai (Taiwan) was nominated as the president in July 2023. This nomination was subsequently ratified during a web meeting held by the core members. As of August 2023, the core team under Dr. Lai's leadership includes Chan Kwon Jung (Korea), Zhiyan Liu (China), Andrey Bychkov (Japan), Radhika Srinivasan (India), Mitsuyoshi Hirokawa (Japan), Somboon Keelawat (Thailand), with Kennichi Kakudo (Japan) serving as a consultant, and Jen-Fan Hang (Taiwan) fulfilling the role of secretary.

There are numerous group studies that are presently under-

Table 3. Articles on “Asian and Western Practice in Thyroid Pathology: Similarities and Differences” in *Gland Surgery*, 2020

| No. | Author(s) | Title | Volume, page |
|-----|---|--|----------------|
| 1 | Kakudo K [14] | Asian and Western practice in thyroid pathology: similarities and differences | 9(5):1614-1627 |
| 2 | Ohori NP [99] | Molecular testing and thyroid nodule management in North America | 9(5):1628-1638 |
| 3 | Kumarasinghe MP [100] | Standardisation of thyroid cytology terminology and practice: are modifications necessary? -a narrative review | 9(5):1639-1647 |
| 4 | Poller DN [101] | Litigation in thyroid cytology and histopathology in England: a very brief overview | 9(5):1648-1652 |
| 5 | Hirokawa M, Suzuki A, Higuchi M, et al. [102] | The Japanese reporting system for thyroid aspiration cytology 2019 (JRSTAC2019) | 9(5):1653-1662 |
| 6 | Ito Y, Miyauchi A [103] | Active surveillance of low-risk papillary thyroid microcarcinomas | 9(5):1663-1673 |
| 7 | Zhu Y, Wu H, Huang B, Shen X, Cai G, Gu X [104] | <i>BRAFV600E</i> mutation combined with American College of Radiology thyroid imaging report and data system significantly changes surgical resection rate and risk of malignancy in thyroid cytopathology practice | 9(5):1674-1684 |
| 8 | Pusztaszeri MP, Tamilia M, Payne RJ [105] | Active surveillance for low-risk small papillary thyroid cancer in North American countries: past, present and future (bridging the gap between North American and Asian practices) | 9(5):1685-1697 |
| 9 | Okamoto T, Omi Y, Yoshida Y, Horiuchi K, Abe K [106] | Radioactive iodine treatment of papillary thyroid carcinoma in Japan | 9(5):1698-1707 |
| 10 | Michael CW, Kameyama K, Kitagawa W, Azar N [107] | Rapid on-site evaluation (ROSE) for fine needle aspiration of thyroid: benefits, challenges and innovative solutions | 9(5):1708-1715 |
| 11 | Liu Z, Sui S, Su P, et al. [108] | The effect of implementing pre-surgical ultrasound-guided fine-needle aspiration biopsy on thyroid surgery, a 6-year interrupted time series analysis in Qilu Hospital of Shandong University | 9(5):1716-1723 |
| 12 | Canberk S [109] | Precursor and borderline lesions of the thyroid (indolent lesions of epithelial origin): from theory to practice | 9(5):1724-1734 |
| 13 | Nguyen TPX, Truong VT, Kakudo K, Vuong HG [64] | The diversities in thyroid cytopathology practices among Asian countries using the Bethesda System for Reporting Thyroid Cytopathology | 9(5):1735-1746 |
| 14 | Oo ZP, Hlaing AM, Kyi KCS, Fukuoka J, Bychkov A [110] | An overview of thyroid fine-needle aspiration practice in Myanmar | 9(5):1747-1753 |
| 15 | Tangnuntachai N, Rangdaeng S, Koonmee S, Tangjaturonrasme N, Keelawat S [111] | Pathological practice and management of thyroid nodules: a Thai perspective | 9(5):1754-1763 |
| 16 | Ooi LY, Nga ME [112] | Atypia of undetermined significance/follicular lesion of undetermined significance: Asian vs. non-Asian practice, and the Singapore experience | 9(5):1764-1787 |
| 17 | Abelardo AD, Sotalbo KCJ [113] | Clinical management of thyroid aspirates diagnosed as atypia of undetermined significance in the Philippines | 9(5):1788-1796 |
| 18 | Guleria P, Mani K, Agarwal S [114] | Indian experience of AUS/FLUS diagnosis: is it different from rest of Asia and the West? -A systematic review and meta-analysis | 9(5):1797-1812 |
| 19 | Odate T, Oishi N, Vuong HG, Mochizuki K, Kondo T [115] | Genetic differences in follicular thyroid carcinoma between Asian and Western countries: a systematic review | 9(5):1813-1826 |
| 20 | Nakra T, Jain D, Agarwal S [116] | Thyroid lymphoproliferative lesions in Asia | 9(5):1827-1837 |
| 21 | Li Y, Inomata K, Nishihara E, Kakudo K [117] | IgG4 thyroiditis in the Asian population | 9(5):1838-1846 |
| 22 | Bai Y, Niu D, Yao Q, Lin D, Kakudo K [118] | Updates in the advances of sporadic medullary thyroid carcinoma: from the molecules to the clinic | 9(5):1847-1856 |
| 23 | Jung CK, Lee S, Bae JS, Lim DJ [119] | Late-onset distant metastases confer poor prognosis in patients with well-differentiated thyroid cancer | 9(5):1857-1866 |
| 24 | Choden S, Keelawat S, Jung CK, Bychkov A [120] | An affordable immunohistochemical approach to estimate the prevalence of <i>BRAFV600E</i> in large cohort studies—establishing the baseline rate of <i>BRAF</i> mutation in an institutional series of papillary thyroid carcinoma from Thailand | 9(5):1867-1877 |
| 25 | Rashid FA, Munkhdelger J, Fukuoka J, Bychkov A [121] | Prevalence of <i>BRAFV600E</i> mutation in Asian series of papillary thyroid carcinoma—a contemporary systematic review | 9(5):1878-1900 |

way, with their findings expected to be published in the near future. The Asian Thyroid Working Group remains committed to offering a collaborative platform for all its members, which is composed of 58 members from 14 countries in September 2023. A notable contribution to the academic community will be the 3rd edition of “Thyroid FNA Cytology, Differential Diagnosis, and Pitfalls,” slated for release in 2023. This edition will encom-

pass an in-depth discussion of all topics presented in the article as mentioned above [52]. The volume will feature more than 60 chapters penned by esteemed Asian authors. Within these chapters, we present a reporting system tailored to the Asian medical context, given that high-cost gene panel tests are not a commonplace practice (as detailed in Table 9).

The core team, along with the project leader, is enthusiastic

Table 4. Pre-Congress Joint Symposium by Working Group of Asian Thyroid FNA Cytology held on March 16, 2017 in Busan, Korea

| Chairpersons | Topic | Presenter |
|---|--|-----------------------------|
| Part 1: Thyroid FNA Cytology in Asian Countries, Current and Future | | |
| SoonWon Hong (Korea) and Chiung-Ru Lai (Taiwan) | Thyroid FNA practice in Korea | SoonWon Hong (Korea) |
| | Cytological-histological correlation studies on thyroid FNA from Thailand | Somboon Keelawat (Thailand) |
| | Thyroid FNA practice in China | Zhiyan Liu (China) |
| | Current status of thyroid FNA cytology in Taiwan | Chiung-Ru Lai (Taiwan) |
| | The Japanese system of thyroid FNA cytology | Shinya Satoh (Japan) |
| | Time travel of an old entity with a new name: NIFTP. A cytomorphologist's perspective and data analyze of institutes from Turkey | Sule Canberk (Turkey) |
| Part 2: Impacts of NIFTP on Thyroid FNA Cytology | | |
| Chan Kwon Jung (Korea) and Mitsuyoshi Hirokawa (Japan) | Proposal of NIFTP and borderline thyroid tumors | Kennichi Kakudo (Japan) |
| | Impact of NIFTP on rates of malignancy for FNA diagnostic categories | Andrey Bychkov (Thailand) |
| | Cytological Findings and Diagnostic Significance of NIFTP and WDT-UMP | Mitsuyoshi Hirokawa (Japan) |
| | Diagnostic criteria of PTC-N, NIFTP and WDT-UMP among Asian pathologists | Zhiyan Liu (China) |
| | Molecular correlates and rate of lymph node metastasis of NIFTP and invasive EFVPTC | Chan Kwon Jung (Korea) |

FNA, fine needle aspiration; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; WDT, well differentiated tumor; UMP, uncertain malignant potential; PTC-N, papillary thyroid carcinoma type nuclear features; EFVPTC, encapsulated follicular variant of papillary thyroid carcinoma.

Table 5. The Working Group of Asian thyroid FNA cytology: recent achievements, current activities, and prospective directions was held on January 19, 2018 in Chiang Mai, Thailand, as the second companion meeting by the Asian Thyroid WG

| Chairpersons | Topic | Presenter |
|---|---|---------------------------------|
| Samreung Rangdaeng (Thailand) and Kennichi Kakudo (Japan) | Thyroid FNA practice in the Philippines | Agustina Abelardo (Philippines) |
| | A survey of current practice of thyroid FNA in Taiwan | Jen-Fan Hang (Taiwan) |
| | Experience of implementing Bethesda system of reporting in BPKIHS, Nepal | Sushil Dhakal (Nepal) |
| | Thyroid FNA in single vs. multiple unit service | Pichet Sampatanukul (Thailand) |
| | Active surveillance for indeterminate thyroid nodules and risk of malignancy | Kennichi Kakudo (Japan) |
| | Diagnostic impact of <i>BRAF</i> testing in thyroid FNA | Ju Yeon Pyo (Korea) |
| | Molecular profile of NIFTP | Chan Kwon Jung (Korea) |
| | How the introduction of NIFTP has changed our practice – Results of survey by the WG | Andrey Bychkov (Thailand) |
| | Where is the NIFTP in thyroid FNA | SoonWon Hong (Korea) |
| | Evaluation of follicular patterned lesions “including oncocytic” by FNA based on the new WHO classification | Sule Canberk (Turkey) |
| | Atypia of undetermined significance: Its cyto-histologic outcome and risk of malignancy | Annette Salillas (Philippines) |
| | Needle tract implantation after thyroid fine needle aspiration procedure | Toshitsugu Hayashi (Japan) |

FNA, fine needle aspiration; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; WHO, World Health Organization.

Table 6. The third Asian Thyroid Working Group Companion Meeting (Asian Practice of Thyroid FNA Cytology) was held on May 8, 2019 at the 20th International Congress of Cytology (ICC Sydney)

| Chairpersons | Topic | Presenter |
|---|--|-----------------------------------|
| Chiung-Ru Lai (Taiwan) and Chan Kwon Jung (Korea) | A welcome message and Introduction of Australian System for Reporting Thyroid FNA Cytology | Pryanthi Kumarasinghe (Australia) |
| | The use of the Bethesda System for Reporting Thyroid Cytopathology in Korea: a nationwide multicenter survey by the Korean Society of Endocrine Pathologists | SoonWon Hong (Korea) |
| | Active surveillance for indeterminate thyroid nodules in China | Yun Zhu (China) |
| | Implementation of the Bethesda system for reporting thyroid cytopathology in Japan | Mitsuyoshi Hirokawa (Japan) |
| | Impact of NIFTP on the Bethesda system in Asian practice | Andrey Bychkov (Thailand) |
| | Hürthle cell lesions in Asian practice: multi-institutional studies in 2018 and 2019 | Deepali Jain (India) |

FNA, fine needle aspiration; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

Table 7. On 16th of November 2019, one more (the 4th face to face meeting) companion meeting was held at the 58th Japanese Society of Clinical Cytology (JSCC) Fall Meeting in Okayama, Japan as the Global Asian Forum

| Chairpersons | Topic | Presenter |
|---|--|------------------------------------|
| Andrey Bychkov (Japan) and Kennichi Kakudo (Japan) | Nuclear features of <i>BRAF</i> mutated PTCs and RAS-mutated neoplasms | Chan Kwon Jung (Korea) |
| | AUS/FLUS in Indian experience with thyroid FNA | Shipra Agarwal (India) |
| | <i>BRAF</i> testing to refine cytology categories: how and when | Priyanthi Kumarasinghe (Australia) |
| | Resection rate and risk of malignancy among TBSRTC categories in Western vs. Asian practice | Huyen-Trang Vu (Vietnam) |
| | Cytological diagnosis of medullary thyroid carcinoma among Asian Working Group in Thyroid Cytology | Chih-Yi Liu (Taiwan) |
| | Observer variation study in the diagnosis of follicular neoplasms | Yaqiong Li (China) |

PTC, papillary thyroid carcinoma; AUS, atypia of undetermined significance; FLUS, follicular lesions of undetermined significance; FNA, fine needle aspiration; TBSRTC, The Bethesda System for Reporting Thyroid Cytopathology.

Table 8. At the 21st International Congress of Cytology held at Baltimore, USA, the 5th face to face companion meeting by the Asian Thyroid Working Group was held on November 18, 2022, as a cytology short course 5: Why Are There Significant Differences Among Us in Thyroid Nodule Practices?

| Chairpersons | Topic | Presenter |
|--|--|-----------------------------|
| Kennichi Kakudo (Japan) and Jen-Fan Hang (Taiwan) | A high diagnostic threshold of RAS-like nuclear features in Asian pathologists impacts thyroid nodule practice | Kennichi Kakudo (Japan) |
| | Diagnoses of <i>BRAF</i> -like PTC and RAS-like PTC | Chan Kwon Jung (Korea) |
| | Molecular testing for cytologically indeterminate thyroid nodules | Jen-Fan Hang (Taiwan) |
| | Japanese thyroid practice using the Bethesda system | Mitsuyoshi Hirokawa (Japan) |

PTC, papillary thyroid carcinoma.

Table 9. Histological type-oriented reporting system of thyroid FNA cytology, useful for ancillary tests

| Cytological category |
|--|
| 1) Inadequate (non-diagnostic) |
| Adequate specimen categories |
| 2) Normal or benign |
| 3) RAS-like (FA/FTC) tumor lineage |
| A1: Favor benign (low risk) |
| A2: Borderline (intermediate risk) |
| A3: Favor malignant (high risk) |
| A4: Suspicious for malignancy (probability of FTC, PDC, etc. should be stated) |
| 4) <i>BRAF</i> -like (PTC) tumor lineage |
| B1: Low risk dysplasia |
| B2: Intermediate risk dysplasia |
| B3: High risk dysplasia (suspicious for PTC) |
| B4: Conclusive PTC type malignancy |
| 5) Other types (unspecified lineage, or other than RAS-like and <i>BRAF</i> -like tumor lineages) |
| C1: Low risk |
| C2: Intermediate risk |
| C3: High risk (probability of C cell carcinoma, ATC, ML and metastatic carcinoma should be stated) |

The original idea of this histological type-oriented reporting system of thyroid FNA cytology was first proposed by Kakudo et al in 2019 [122] and updated in 2023 [52].

FNA, fine needle aspiration; RAS, rat sarcoma virus; FA, follicular adenoma; FTC, follicular thyroid carcinoma; PDC, poorly differentiated carcinoma; PTC, papillary thyroid carcinoma; *BRAF*, v-Raf murine sarcoma viral oncogene homolog B; ATC, anaplastic thyroid carcinoma; ML, malignant lymphoma.

about creating more avenues for budding Asian pathologists and cytopathologists to engage with the broader international pathology community. We ardently hope that the activities under the aegis of the Asian Thyroid Working Group will act as a springboard for the younger generation, integrating them with the global community and bolstering their careers in pathology and cytopathology. A list of publications by the Asian Thyroid Working members from 2017 to 2023 is available in Supplementary Table S1.

Supplementary Information

The Data Supplement is available with this article at <https://doi.org/10.4132/jptm.2023.10.04>.

Ethics Statement

Not applicable.

Availability of Data and Material

A publication list by Asian WG member is available as a Supplementary Table S1.

Code Availability

Not applicable.

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Conflicts of Interest

C.K.J., the editor-in-chief, along with K.K., Z.L., A.B., and C.-R.L., who are contributing editors of the *Journal of Pathology and Translational Medicine*, were not involved in the editorial evaluation or decision to publish this article. All other authors have declared no conflicts of interest.

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Senescent tumor cells in colorectal cancer are characterized by elevated enzymatic activity of complexes 1 and 2 in oxidative phosphorylation

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Background: Cellular senescence is defined as an irreversible cell cycle arrest caused by various internal and external insults. While the metabolic dysfunction of senescent cells in normal tissue is relatively well-established, there is a lack of information regarding the metabolic features of senescent tumor cells. **Methods:** Publicly available single-cell RNA-sequencing data from the GSE166555 and GSE178341 datasets were utilized to investigate the metabolic features of senescent tumor cells. To validate the single-cell RNA-sequencing data, we performed senescence-associated β -galactosidase (SA- β -Gal) staining to identify senescent tumor cells in fresh frozen colorectal cancer tissue. We also evaluated nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase (NADH-TR) and succinate dehydrogenase (SDH) activity using enzyme histochemical methods and compared the staining with SA- β -Gal staining. MTT assay was performed to reveal the complex 1 activity of the respiratory chain in in-vitro senescence model. **Results:** Single-cell RNA-sequencing data revealed an upregulation in the activity of complexes 1 and 2 in oxidative phosphorylation, despite overall mitochondrial dysfunction in senescent tumor cells. Both SA- β -Gal and enzyme histochemical staining using fresh frozen colorectal cancer tissues indicated a high correlation between SA- β -Gal positivity and NADH-TR/SDH staining positivity. MTT assay showed that senescent colorectal cancer cells exhibit higher absorbance in 600 nm wavelength. **Conclusions:** Senescent tumor cells exhibit distinct metabolic features, characterized by upregulation of complexes 1 and 2 in the oxidative phosphorylation pathway. NADH-TR and SDH staining represent efficient methods for detecting senescent tumor cells in colorectal cancer.

Key Words: Colorectal neoplasms; Metabolism; Cellular senescence; Oxidative phosphorylation; NADH

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Cellular senescence is an irreversible cell cycle arrest first reported in normal fibroblasts after serial cultivation in vitro by Hayflick and Moorhead in 1961 [1]. Cellular senescence can be induced by a variety of factors, including telomere shortening or dysfunction, DNA damage, oncogene activation or loss of tumor suppressor functions, epigenetic changes, and organelle damage [2,3]. Moreover, cellular senescence can be induced by oncogenic activation, a process known as oncogene-induced senescence (OIS) [4]. Since OIS was initially observed in premalignant le-

sions, it was initially considered to serve as a barrier to malignant transformation [4]. However, recent studies have demonstrated that senescent malignant cells can still be identified in various types of cancers [5-8]. Furthermore, these senescent cancer cells have been shown to promote the development and/or progression of cancer [5-7].

Despite being in a state of permanent proliferation arrest, senescent cells remain metabolically active [9,10]. Indeed, senescent cells are characterized by its vigorous synthesis of specific

proteins: various cytokines and release factors, which are called senescence-associated secretory phenotype (SASP) [11]; the presence of senescent cells may massively affect adjacent microenvironment by inducing inflammatory responses [11]. Therefore, senescent cells may need sufficient energy to fit their demand to synthesize various metabolites. Since mitochondrial dysfunction is primarily involved in cellular senescence, the efficiency of oxidative phosphorylation (OXPHOS) in senescent cells from normal tissues is significantly decreased [12]. Therefore, it is widely accepted that senescent cells maintain their energy demand by increasing glycolysis and fermentation [13]. However, multiple studies have revealed that the OXPHOS status in malignant cells can vary. Some studies have shown that cancer cells exhibit upregulated glycolysis compared to normal cells, and OXPHOS is commonly downregulated in various cancers [14,15], while others have shown that OXPHOS can increase [16-18]. Nevertheless, the status of OXPHOS in senescent tumor cells remains unknown. From this background, we hypothesized that the main energy production pathway of senescent tumor cells might be different from non-senescent tumor cells. To check this hypothesis, we utilized single-cell RNA-sequencing to reveal the metabolic feature of senescent tumor cells. Subsequently, enzymatic activity related with OXPHOS was examined; enzyme histochemical methods, such as nicotinamide adenine dinucleotide dehydrogenase–tetrazolium reductase (NADH-TR) and succinate dehydrogenase (SDH) staining, were applied. By visualizing the activity of these enzymes, NADH-TR and SDH staining can provide valuable insights into the metabolic state of cells. The main purpose of the study is to check the correlation between senescent tumor cells and enzymatic activity related with OXPHOS in colorectal cancer (CRC) cells.

MATERIALS AND METHODS

CRC sample preparation

We obtained CRC tissue samples from pathological specimens of CRC patients who underwent surgery at Ajou University Hospital. An experienced pathologist sampled fresh tissues from both normal and tumor areas immediately after resection. We included patients who had not received chemotherapy or radiotherapy before surgery. Cases that failed to undergo any one of the three staining procedures for senescence and mitochondrial OXPHOS were previously excluded. Out of the 35 CRC cases, 28 met the inclusion criteria.

Single-cell RNA-sequencing

Publicly available CRC single-cell RNA-sequencing (scRNA-seq) dataset, GSE166555 and GSE178341 were used for analysis [19,20]. Among patients, patient #8 and #C103 who markedly expressed *CDKN2A* was used for analysis to minimize the batch effect, respectively. *CDKN2A*⁻ and *CDKN2A*⁺ cancer cells were analyzed and compared for further analysis. Detailed information about preprocessing steps and cancer cell extraction is described in our previous study [8].

Senescence-associated β -galactosidase staining

For senescence-associated β -galactosidase (SA- β -Gal) staining, representative fresh CRC tissue samples were immediately frozen at -20°C using a cryostat (Leica Biosystems, Nußloch, Germany) after being coated with OCT compound (Sakura Finetek, Torrance, CA, USA). Fresh frozen sections with a thickness of $6\ \mu\text{m}$ were placed on slides. After fixation with phosphate buffer saline (PBS) buffer containing formaldehyde for 1 minute, the sections were incubated in a mixture containing 1 mg/mL of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (Bioneer, Daejeon, Korea), 40 mM citric acid/sodium phosphate buffer at pH 5.8, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl_2 at 37°C in an incubator for 14 hours. Following incubation, the slides were washed three times with PBS. Counterstaining was carried out with nuclear fast red solution at room temperature for approximately 5 minutes. After the counterstaining was complete, the slides were mounted using mounting solution (Sakura Finetek). All slides with SA- β -Gal staining were scanned at a minimum of $20\times$ magnification using an Aperio AT2 slide scanner (Leica Biosystems).

NADH-TR staining

For NADH-TR staining, fresh frozen section slides were incubated in a mixture of nitro tetrazolium blue chloride solution (2 mg/mL NBT/0.05 M Tris buffer pH 7.6; Sigma-Aldrich, St. Louis, MO, USA) and reduced nicotinamide adenine dinucleotide solution (1.6 mg/mL; NADH/0.05 M Tris buffer pH 7.6; Sigma-Aldrich). The sections were then incubated at 37°C in an incubator for 2 hours. After incubation, the slides were washed three times with PBS and counterstained with nuclear fast red solution at room temperature for approximately 5 minutes. Once the counterstaining was complete, the slides were mounted using mounting solution (Sakura Finetek). All slides with NADH staining were scanned at a minimum of $20\times$ magnification using an Aperio AT2 slide scanner (Leica Biosystems).

SDH staining

For SDH staining, fresh frozen section slides were incubated in a mixture containing 0.2 M phosphate buffer at pH 7.4, 0.1 M MgCl₂, 0.2 M succinic acid (Sigma-Aldrich), and 2.4 mM nitro tetrazolium blue chloride (Sigma-Aldrich). The sections were incubated at 37°C in an incubator for 2 hours. After incubation, the slides were washed three times with PBS and counterstained with nuclear fast red solution at room temperature for approximately 5 minutes. Once the counterstaining was complete, the slides were mounted using mounting solution (Sakura Finetek). All slides with SDH staining were scanned at a minimum of 20× magnification using an Aperio AT2 slide scanner (Leica Biosystems).

Evaluation of OXPHOS in CRC tissues

To investigate senescent cells in CRC, we analyzed virtual slides of SA-β-Gal staining in CRC tissue sections. SA-β-Gal positivity was identified in cancer cells, stromal cells, and/or macrophages. We specifically assessed the percentages of SA-β-Gal positive cancer cell areas relative to the total cancer cell areas. For the analysis of OXPHOS in CRC, we examined virtual slides of NADH-TR and SDH staining in CRC tissue sections. We compared the intensity of purple-blue formazan pigments in cancer cells to that in normal epithelial cells or stromal cells and considered it positive if the intensity was similar or more intense. We also evaluated the percentages of positive cancer cell areas within the total cancer cell areas. Furthermore, we compared the patterns of positivity in SA-β-Gal, NADH-TR, and SDH staining in CRC tissue sections. We conducted further analysis to determine the association between the positivity of SA-β-Gal, NADH-TR, and SDH staining and clinicopathologic parameters of CRC patients.

Cell culture and hydrogen peroxide treatment

CRC cell lines SNU254 and SNU1544 were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained in RPMI supplemented with 10% fetal bovine serum. For the hydrogen peroxide treatment experiment, cells were treated with 100 μM and 200 μM H₂O₂/dimethyl sulfoxide solution. After treatment for the indicated times, cells were incubated at 37°C for 2 days. The cells were then washed with PBS buffer and subjected to measurement of hydrogen peroxide using live cell imaging microscopy.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Control and hydrogen peroxide-treated SNU254 and SNU1544

cells were preincubated at a concentration of 1×10^6 cells/mL in culture medium with 1 μg/mL actinomycin C1 for 3 hours at 37°C and 5% CO₂. Cells were seeded at a concentration of 5×10^4 cells/well in 100 μL culture medium containing 1 μg/mL actinomycin C1 into microplates. Cell cultures were incubated for 24 hours at 37°C with 5% CO₂. After the incubation period, MTT labeling reagent (final concentration 0.5 mg/mL) was added to each well. The microplate was incubated for 4 hours in a humidified atmosphere. Solubilization solution was added into each well followed by overnight incubation in the incubator in a humidified atmosphere. Complete solubilization of the purple formazan crystals was checked and the absorbance of the samples was measured using a microplate reader. The wavelength to measure absorbance of the formazan product was 600 nm.

Statistical analysis

The statistical analysis was conducted using IBM SPSS ver. 25.0 (IBM Corp., Armonk, NY, USA). The Student independent t test or Fisher's exact tests were used to analyze the parameters. A linear regression was performed in Prism 9 software (GraphPad Software, San Diego, CA, USA). A p-value of less than or equal to .05 was considered statistically significant. For adjusted p-values in gene set enrichment analysis (GSEA), the raw p-values were ranked in ascending order. The adjusted p-values are calculated for each gene set using the ranked raw p-values and the formula of the Benjamin-Hochberg procedure.

RESULTS

Metabolic features of senescent tumor cells

To elucidate the metabolic features of senescent tumor cells, we employed scRNA-seq using the publicly available CRC dataset, GSE166555 [19]. To minimize the batch effect, we exclusively analyzed patients who exhibited marked expression of p16^{INK4A} (*CDKN2A*), as described in our previous study. Among the 274 *CDX2* and *EPCAM* double-positive cancer cells, 198 cells tested positive for *CDKN2A*, while 76 cells tested negative (Fig. 1A). To validate the senescence-related phenotype of *CDKN2A*-positive CRC cells, we conducted GSEA. GSEA clearly demonstrated that *CDKN2A*-positive cells exhibit senescent phenotypes (Fig. 1B). Numerous prior studies have already reported that mitochondrial dysfunction is a hallmark of senescence in primary cells from normal tissues [21-23]. However, it remains unknown in the context of senescent tumor cells. Similar to senescent cells from normal tissues, senescent tumor cells exhibit OXPHOS dysfunction (Fig. 1C). GSEA also revealed a sig-

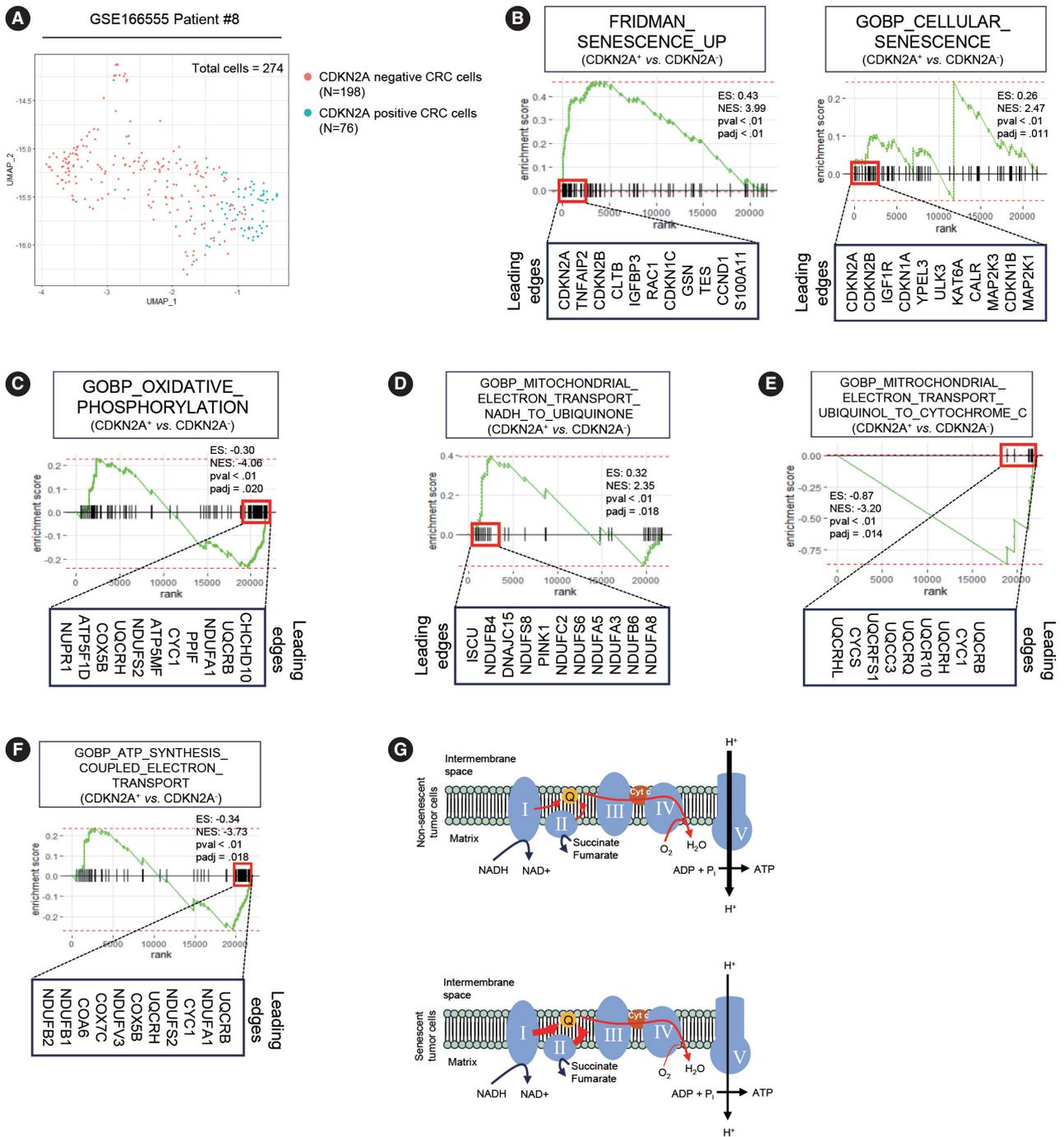


Fig. 1. Single-cell RNA-sequencing (scRNA-seq) of human colorectal cancer tissues. (A) Uniform Manifold Approximation and Projection (UMAP) of public scRNA-seq dataset GSE166555 patient #8 who markedly expressed *CDKN2A* is shown. A total of 274 cells were analyzed. (B) Gene set enrichment analysis (GSEA) of “Fridman Senescence Up” (left panel) and “GOBP Cellular Senescence” gene sets was performed in *CDKN2A*⁺ vs. *CDKN2A*⁻ cancer cells. (C) GSEA of a “GOBP Oxidative Phosphorylation” gene set was performed in *CDKN2A*⁺ vs. *CDKN2A*⁻ cancer cells. (D) GSEA of a “GOBP Mitochondrial Electron Transport NADH to Ubiquinone” gene set was performed in *CDKN2A*⁺ vs. *CDKN2A*⁻ cancer cells. (E) GSEA of a “GOBP Mitochondrial Electron Transport Ubiquinol to Cytochrome c” gene set was performed in *CDKN2A*⁺ vs. *CDKN2A*⁻ cancer cells. (F) GSEA of a “GOBP ATP Synthesis Coupled Electron Transport” gene set was performed in *CDKN2A*⁺ vs. *CDKN2A*⁻ cancer cells. (G) Schematic image of expected electron transport efficiency in senescent and non-senescent tumor cells, respectively. The thickness of red arrows indicates the efficiency of electron flow. ‘*padj*’ indicates the adjusted p-value. CRC, colorectal cancer; ES, enrichment score; NES, normalized enrichment score.

nificant upregulation of the electron transport from NADH to ubiquinone in senescent tumor cells (Fig. 1D). However, the electron transport from ubiquinol to cytochrome c was inhibited in senescent tumor cells (Fig. 1E). Furthermore, ATP synthesis was downregulated in senescent tumor cells (Fig. 1F). These findings were validated in another scRNA-seq dataset, GSE178341 (Supplementary Fig. S1) [20]. Therefore, it is highly suggested that the upregulation of complexes 1 and 2 of OXPHOS in senescent tumor cells is a result of a potential feedback loop caused by dysfunctions in the subsequent electron transport pathways (Fig. 1G).

Patient characteristics according to the SA- β -Gal positivity

To confirm the metabolic phenotype suggested by the scRNA-seq results, we performed enzymatic histochemistry analysis using NADH-TR and SDH. NADH is a component of complex 1 of the electron transport chain, which is part of OXPHOS [24]. In contrast, SDH is a component of complex 2 of OXPHOS [24]. However, there was a challenge in performing p16^{INK4A} immunostaining because it is typically performed on formalin-fixed paraffin-embedded (FFPE) CRC samples, while enzymatic histochemistry can only be conducted on fresh or frozen tissues. To address this issue, we employed SA- β -Gal staining, another standard senescence marker that can be utilized in frozen tissues. Previously, we had confirmed the correlation between p16^{INK4A} and SA- β -Gal staining in CRC cells [7,8]. Therefore, following analysis was performed based on the SA- β -Gal staining rather than immunostaining. A wide variation in the number of SA- β -Gal-positive cells were observed among CRC samples. These cells were distributed not only in the cancerous epithelium but occasionally in the normal epithelium (Supplementary Fig. S2), but also in stromal fibroblasts, and macrophages [2,7]. In this study, SA- β -Gal-positive malignant epithelial cells were exclusively examined to analyze the traits of senescent tumor cells in each CRC sample. The proportion of SA- β -Gal staining ranged from less than 1% to more than 90% across the CRC cases. We divided the CRC samples into two groups based on the proportion of SA- β -Gal-positive cancer cells: a low SA- β -Gal group (less than 30%) and a high SA- β -Gal group (more than 30%). The clinicopathologic characteristics of patients according to the SA- β -Gal group were shown in Table 1. We did not find any significant associations between clinicopathologic characteristics and the low or high SA- β -Gal-positive groups.

NADH-TR and SDH staining in senescent tumor cells in vivo

To assess the OXPHOS activity in senescent tumor cells, we

Table 1. Clinicopathologic characteristics of CRC according to the SA- β -Gal staining intensity

| | SA- β -Gal ^{low} (n = 13) | SA- β -Gal ^{high} (n = 15) | p-value |
|--------------------------|---|--|-------------------|
| Age (yr) | 65.7 (43–81) | 64.3 (43–89) | .770 ^a |
| Male sex | 6 (46.2) | 8 (53.3) | .705 ^b |
| Left side tumor | 9 (69.2) | 8 (53.3) | .390 ^b |
| Tumor size (cm) | 4.9 | 5.5 | .420 |
| SA- β -Gal (%) | 24.3 (1–30) | 70.7 (40–90) | <.01 ^a |
| T staging | | | .412 ^b |
| pT1 | 0 | 1 (6.3) | |
| pT2 | 1 (8.3) | 0 | |
| pT3 | 11 (83.3) | 11 (75) | |
| pT4 | 1 (8.3) | 3 (18.8) | |
| N staging | | | .800 ^b |
| pN0 | 5 (38.4) | 7 (46.7) | |
| pN1 | 4 (30.8) | 3 (20.0) | |
| pN2 | 4 (30.8) | 5 (33.3) | |
| M staging | | | .916 ^b |
| M0 | 12 (92.3) | 14 (93.3) | |
| M1 | 1 (7.7) | 1 (6.7) | |
| Tumor differentiation | | | .650 |
| Well | 1 (7.7) | 3 (20.0) | |
| Moderate | 11 (84.6) | 11 (73.3) | |
| Poor | 1 (7.7) | 1 (6.7) | |
| KRAS mutation | 7 (53.8) | 8 (53.3) | .978 ^b |
| NRAS mutation | 0 | 0 | >.99 ^b |
| Microsatellite stability | | | .343 ^b |
| MSI-L–MSS | 13 (100) | 14 (93.3) | |
| MSI-high | 0 | 1 (6.7) | |

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

CRC, colorectal cancer; SA- β -Gal, senescence-associated β -galactosidase; MSI, microsatellite instability; MSI-L, low microsatellite instability; MSS, microsatellite stable.

^ap-value is obtained using Student's t test; ^bp-value is obtained using chi-square test.

conducted NADH-TR staining (Supplementary Fig. S3) and SDH staining (Supplementary Fig. S4) on consecutive fresh frozen CRC tissue sections. Additionally, we performed SA- β -Gal staining and enzymatic histochemical analysis on serial sections of frozen tissues. Our findings revealed a consistent correlation between SA- β -Gal staining and both NADH-TR and SDH staining. The intensity of NADH-TR and SDH staining was found to be higher in cancer tissues with high SA- β -Gal activity (Fig. 2). Conversely, cancer tissues with low SA- β -Gal activity exhibited lower NADH-TR and SDH staining intensity (Fig. 3). A linear regression analysis clearly showed that there is a positive correlation between SA- β -Gal and NADH-TR/SDH staining (Fig. 4), suggesting that complex 1 and 2 activities of OXPHOS in senescent tumor cells are upregulated despite of the mitochondrial dysfunctions.

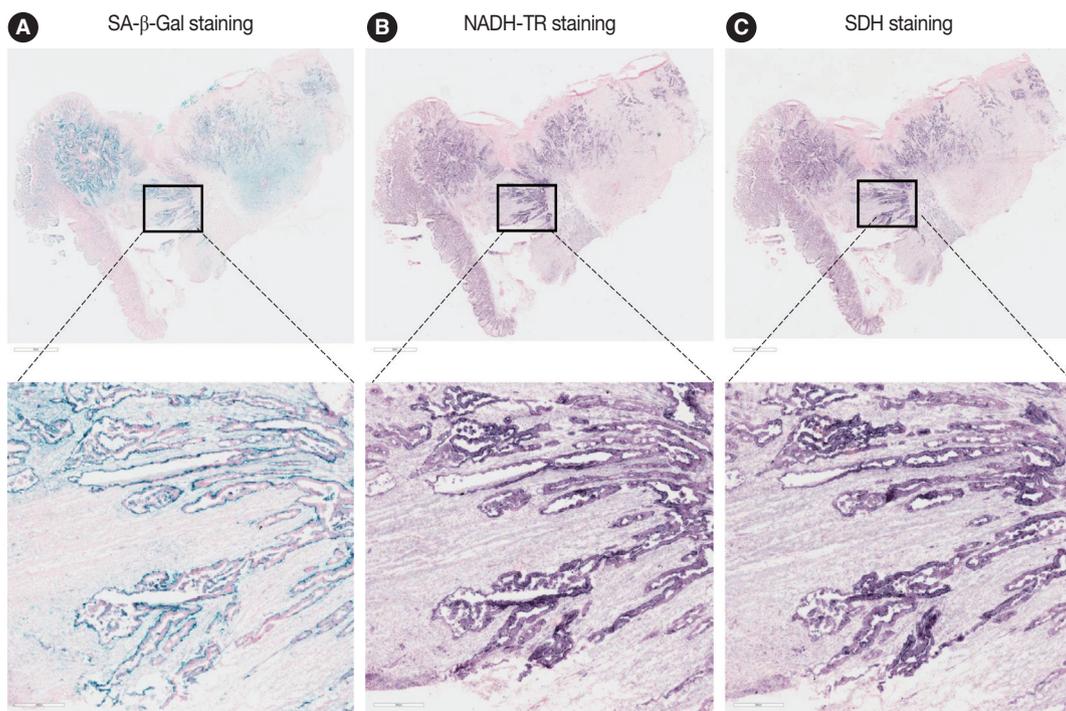


Fig. 2. Nicotinamide adenine dinucleotide dehydrogenase–tetrazolium reductase (NADH-TR) and succinate dehydrogenase (SDH) staining results in the senescence-associated β -galactosidase (SA- β -Gal) high patient. The staining results of SA- β -Gal (A), NADH-TR (B), and SDH staining (C) are shown in the SA- β -Gal high patient.

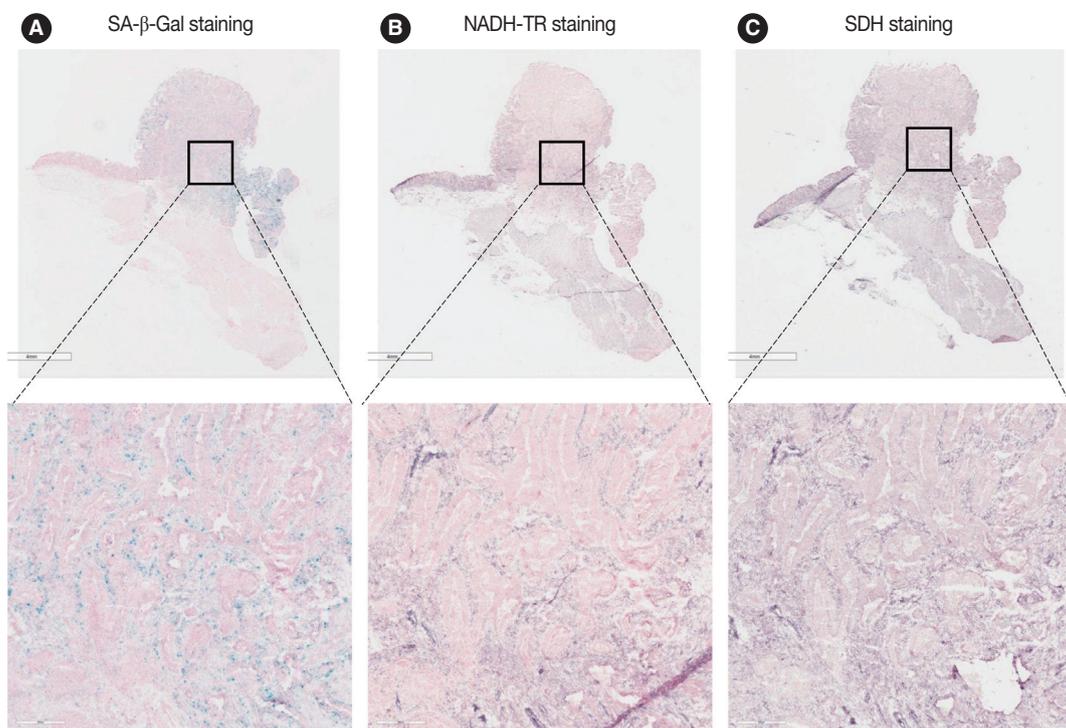


Fig. 3. Nicotinamide adenine dinucleotide dehydrogenase–tetrazolium reductase (NADH-TR) and succinate dehydrogenase (SDH) staining results in the senescence-associated β -galactosidase (SA- β -Gal) low patient. The staining results of SA- β -Gal (A), NADH-TR (B), and SDH staining (C) are shown in the SA- β -Gal low patient.

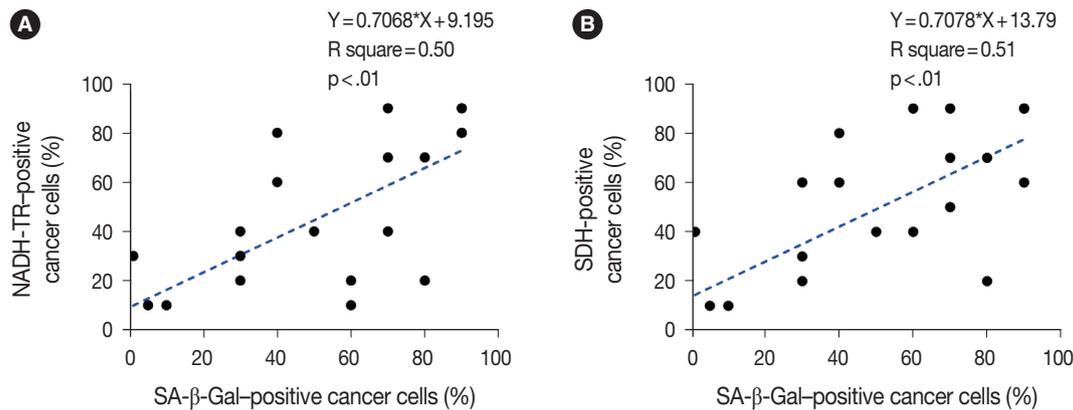


Fig. 4. Scatter plots according to the nicotinamide adenine dinucleotide dehydrogenase–tetrazolium reductase (NADH-TR) (A) and succinate dehydrogenase (SDH) (B) staining results compared with senescence-associated β-galactosidase (SA-β-Gal) staining. p-values are obtained using the hypothesis test for regression slope.

MTT assay in control and senescent tumor cells in vitro

MTT is a widely used method for assessing the viability of cultured cells [25]. Additionally, MTT can be utilized to evaluate mitochondrial function. MTT is reduced to formazan, which strongly absorbs light in the visible range and has low solubility in water [26]. Notably, MTT can be directly reduced by complex I of the respiratory chain, providing a measure of complex I activity in the mitochondria [26]. To assess the complex I activity of senescent tumor cells in vitro, senescence in CRC cell lines SNU1544 and SNU254 was induced by hydrogen peroxide treatment, confirmed by SA-β-Gal staining (Supplementary Fig. S5A). The mRNA levels of representative SASPs were assessed, and SASPs were found to be increased in hydrogen peroxide-treated cancer cells (Supplementary Fig. S5B). MTT assays revealed an upregulation of complex I activity in senescent tumor cells in vitro (Supplementary Fig. S5C), which is consistent with in vivo findings.

DISCUSSION

Cancer cells, owing to their rapid proliferation rate and heightened metabolic activity, employ distinctive energy production mechanisms to meet their energy requirements [27]. Warburg originally observed the phenomenon of markedly increased glucose uptake in cancer cells, now widely recognized as the ‘Warburg effect’ [28,29]. Although Warburg suggested that elevated glycolysis in cancer cells resulted from mitochondrial defects [29], recent studies have shown that the mitochondrial pathway in cancer cells may still be partially functional and contribute to energy production [30]. Consequently, mitochondrial metabolism remains an attractive target for cancer therapy, with numerous

investigations exploring this avenue [31,32]. Nevertheless, a notable challenge in the development of agents targeting mitochondrial metabolism is the resemblance between the metabolic processes of cancer cells and cytotoxic T cells [32–34]. The use of these agents to target cancer cells may inadvertently lead to the elimination of cancer-fighting cytotoxic T cells, potentially exacerbating a prognosis [30,32]. Therefore, the pursuit of metabolism-targeting agents specific to cancer cells represents an emerging frontier in cancer treatment.

Research into cancer metabolism has made significant progress in understanding non-senescent tumor cells [30]. However, the metabolic characteristics of senescent tumor cells remain less clear. Cellular senescence is widely recognized to be closely associated with mitochondrial dysfunction [35]. Although there is ongoing debate about whether cellular senescence induces mitochondrial dysfunction [36] or vice versa [37], it is evident that there is a positive correlation between cellular senescence and mitochondrial dysfunction [38]. Indeed, mitochondrial dysfunction has been observed in various cellular senescence models. Nelson and colleagues demonstrated a link between replicative senescence and mitochondrial dysfunction [39]. Moiseeva and colleagues reported a correlation between OIS and mitochondrial dysfunction [23]. Furthermore, Yoon et al. [21] revealed that the induction of senescence through iron chelation resulted in a reduction in complex 2 activity, preceding the onset of p27kip1-mediated cell cycle arrest in both Chang and normal liver cells. Likewise, senescence induced by transforming growth factor β1 in Mv1Lu lung epithelial cells involved the inhibition of complex 4, leading to the generation of mitochondrial reactive oxygen species [22]. To the best of our knowledge, this is the first study to analyze the metabolic features of senescent cancer cells

in CRC. In our results, senescent tumor cells exhibited relatively more significant defects in OXPHOS compared to non-senescent tumor cells, as indicated by impaired electron transport to cytochrome c and reduced ATP production. This is the same line with the various studies based on multiple senescence models using normal cells [21-23]. However, intriguingly, there was a notable upregulation in the activity of electron transport from NADH to ubiquinone, a function typically associated with complex 1 [24]. While further direct evidence is required, these data suggest that electron transport from ubiquinone to complex 3 may be compromised in senescent tumor cells, leading to an upregulation in the gene expression of complex 1 and 2 as part of a potential feedback loop.

NADH-TR and SDH staining are enzyme histochemical methods that are widely used to examine the oxidative activity of cells, particularly in muscle biopsy. These stains can reveal the oxidative capacity of muscle fibers and their architectural changes in some muscle disorders. NADH and SDH are components of complex 1 and 2 of the electron transport chain, respectively. By visualizing the activity of these enzymes, NADH-TR and SDH staining can provide valuable insights into the metabolic state of cells [40]. In addition to diagnosing muscle disorders, these methods can be applied to screen the OXPHOS status of cancer cells as well as senescent cells. OXPHOS is an important metabolic process not only in cells that drive cancer drug resistance [41], but also in senescent cells associated with the aging process [9]. Therefore, NADH-TR and SDH staining can be used to identify the metabolic state of these cells and provide insights into their response to anticancer therapy or anti-aging therapy.

While cellular senescence in cancer cells has traditionally been considered a defense mechanism against cancer progression [42], recent studies have revealed that senescent tumor cells can actually promote cancer progression by influencing the tumor microenvironment [5-7]. In fact, senescent tumor cells themselves exhibit a higher invasion ability due to the secretion of various extracellular matrix-modulating proteins, such as matrix metalloproteinases [2]. As a result, senescent tumor cells are often located at the invasive front of the cancer tissue rather than its center [5,7]. Moreover, the reversal of the halted proliferation of senescent tumor cells may be associated with cancer relapse in distant metastatic organs. Therefore, the detection of senescent tumor cells is crucial for predicting patient prognosis and potential cancer relapse in the near future. However, there are limited methods available for the detection of senescent tumor cells. Furthermore, many markers for cellular senescence are tumor suppressor genes, such as p53, p21^{Waf1}, and p16^{INK4A} [2,43]. Conse-

quently, they have a higher mutation rate, which can result in the overexpression of malfunctioning proteins due to potential feedback loops [2,44,45]. To overcome this challenge, additional senescence markers, such as a decrease in lamin B1 or detection of H3K9-trimethylation, are utilized [46]. In our experience, the staining of multiple senescence markers using serial sections, combined with CDK inhibitors, and accessory senescence markers are essential to minimize false-positive results when detecting senescent tumor cells in FFPE tissues [2]. While SA- β -Gal is considered a reliable marker for detecting senescent tumor cells in fresh or frozen CRC tissues [47], it often produces false positives due to its inherent β -galactosidase activity [48]. In our study, we found that enzymatic histochemistry using NADH-TR and SDH staining can effectively identify senescent tumor cells in CRC. Therefore, utilizing NADH-TR and SDH staining in conjunction with SA- β -Gal staining, using serial sections, represents a valuable approach to reduce the risk of false positivity associated with SA- β -Gal staining alone.

There are several limitations in this study. Firstly, the results mainly rely on scRNA-seq, enzyme histochemistry analysis, and in vitro MTT assay, which provide relatively indirect evidence compared to various mitochondrial supercomplex assays [39]. Therefore, to confirm the specific dysfunction of the mitochondrial electron transport system, various assays from complex 1 to 4 should be conducted, which will be part of our future studies. Enzyme histochemistry along with functional assays for electron transport complexes may address overall efficiency of the electron transport and OXPHOS system. However, despite similar results in different scRNA-seq datasets in our study consistently indicating that complex 1 and 2 are functionally upregulated in senescent tumor cells [19,20], the reasons for this phenomenon remain unknown. Secondly, the number of patients analyzed in this study was relatively small. Therefore, a larger number of patients will be needed to strengthen the results.

Supplementary Information

The Data Supplement is available with this article at <https://doi.org/10.4132/jptm.2023.10.09>.

Ethics Statement

This study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-BMR-OBS-16-218). All patients provided informed consent.

Availability of Data and Material

The datasets generated or analyzed during the current study are available in the Gene expression Omnibus (GEO) repository from National Center for Biotechnology Information (NCBI) with accession number GSE166555 and GSE178341. Persistent weblinks are <https://www.ncbi.nlm.nih.gov/>

geo/query/acc.cgi?acc=GSE166555 and <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178341>.

Code Availability

Not applicable.

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Conflicts of Interest

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

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Elevated expression of Axin2 in intestinal metaplasia and gastric cancers

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Background: The Wnt signaling pathway regulates crucial cellular processes, including stem cell development and tissue repair. Dysregulation of this pathway, particularly β -catenin stabilization, is linked to colorectal carcinoma and other tumors. Axin2, a critical component in the pathway, plays a role in β -catenin regulation. This study examines Axin2 expression in normal gastric mucosa and various gastric pathologies. **Methods:** Formalin-fixed and paraffin-embedded tissue samples from normal stomach, gastritis, intestinal metaplasia (IM), and gastric carcinoma were collected. Axin2 and β -catenin expression were evaluated using RNA in situ hybridization and immunohistochemistry, respectively. Histo-scores (H-scores) were calculated to quantify expression levels of Axin2. Associations between Axin2 expression and clinicopathological variables were examined. **Results:** Axin2 expression was examined in normal stomach, gastritis, and IM tissues. Axin2 expression was mainly observed in the surface and isthmus areas in the normal stomach and gastritis, whereas Axin2 expression was markedly higher at the bases of IM. Axin2 H-scores were significantly elevated in IM (mean \pm standard deviation [SD], 87.0 ± 38.9) compared to normal (mean \pm SD, 18.0 ± 4.5) and gastritis tissues (mean \pm SD, 33.0 ± 18.6). In total, 30% of gastric carcinomas showed higher Axin2 expression. Axin2 expression did not have significant associations with age, sex, Lauren classification, histological differentiation, invasion depth, and lymph node metastasis. However, a strong positive correlation was observed between Axin2 and nuclear β -catenin in gastric carcinomas ($p < .001$). **Conclusions:** Axin2 expression was significantly increased in IM compared to normal and gastritis cases. In addition, Axin2 showed a strong positive association with nuclear β -catenin expression in gastric carcinomas, demonstrating a close relationship with abnormal Wnt/ β -catenin signaling pathway.

Key Words: Wnt signaling pathway; Axin2; β -catenin; Gastric carcinoma; Intestinal metaplasia

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Wnt signaling serves a variety of critical functions in the human body, orchestrating the regulation of stem cell development, cell differentiation, proliferation, immune cells and tissue repair and regeneration [1,2]. Delicate and various communication mechanisms exist between the Wnt protein and a receptor called Fizzled, to facilitate the precise execution of biological roles in a coordinated manner. Wnt signaling is classified into canonical and noncanonical pathways [3]. Inappropriate activation of the canonical Wnt signaling pathway contributes to colorectal carcinoma and a variety of other tumors [4]. The stabilization of β -catenin is a key regulatory step during cell fate changes and transformations to tumor cells [5].

The axis inhibition protein 2 (Axin2) is a crucial component of the cytoplasmic complex that targets β -catenin for degradation in the absence of ligand [6]. In the absence of Wnt ligand,

cytoplasmic β -catenin is degraded; degradation depends on a destruction complex that includes Axin2, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase-3 β (GSK-3 β). When Wnt proteins bind the Frizzled and LRP5/6 coreceptors, Axin2 is removed from the destruction complex, and stable β -catenin moves to the nucleus where, in combination with LEF/TCF DNA binding proteins, it activates target gene expression. In the presence of Wnt ligand, the Wnt signaling pathway induces dephosphorylation of Axin2, and the dephosphorylated Axin2 binds β -catenin less efficiently compared to its phosphorylated form [5]. The molecules that inhibit the activity of tankyrase reduce Wnt signaling in carcinoma cell lines, and it has been suggested that they provide a new option for therapy for Wnt-based tumors [7]. Axin2 mutation have been reported in a variety of human carcinomas including hepatocel-

lular carcinomas and colorectal carcinomas [8-10].

Axin2 operates as a scaffold protein within the Wnt signaling pathway [11]. Prior investigations have explored Axin2 mutations in connection with gastric carcinoma (GC) [12]. Nonetheless, the differences in Axin2 expression, encompassing both the spatial distribution and intensity, among normal tissue, gastritis, intestinal metaplasia (IM), and GC, have not been investigated. In this study, we aim to thoroughly examine the expression of Axin2 in various gastric pathologies and particularly evaluate its significance in GC.

MATERIALS AND METHODS

Tissue samples

The samples were collected from patients with gastric carcinomas who underwent surgical resection at Jeju National University Hospital (JNUH) in Jeju, Korea, between 2012 and 2017, including GC (n = 56), IM (n = 10), chronic gastritis (n = 5), and normal (n = 5) gastric samples. Inflammation in the gastric mucosa was graded according to the Sydney classification and we considered the cases as chronic gastritis when inflammation grade is moderate or marked. The classification of histological subtypes of carcinomas was independently determined by two pathologists (D.H.L. and B.J.). For GC cases, we gathered clinical and pathological data, including age, sex, Lauren classification, histological grade, invasion depth, and lymph node metastasis.

Tissue array construction

Total five tissue microarrays (TMAs) were constructed; four TMAs included 96 cores from GC cases and 20 cores from non-cancerous gastric lesions. In brief, representative tumor portion was carefully selected from hematoxylin and eosin-stained slides through histologic examination. Each representative tumor portion comprised more than 70% of the cell population. The 4-mm diameter core tissues were obtained from individual GC paraffin blocks and non-cancerous block. Then, these core tissues were arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea).

Immunohistochemistry and interpretation

Immunohistochemistry (IHC) targeting β -catenin was conducted on 4- μ m sections of the TMAs. IHC was done with the BOND-MAX automated immunostainer and a Bond Polymer Refine Detection kit (Leica Microsystems, Wetzlar, Germany) according to the manufacturer's guidelines. The primary antibody used was anti- β -catenin (1:800, catalog number 17C2, Novo-

castra Laboratories, Newcastle, UK). Positive nuclear β -catenin staining was defined as cases where more than 10% of tumor cell nuclei displayed strong staining for β -catenin.

Axin2 RNA in situ hybridization and interpretation

To detect Axin2 mRNA, we applied RNAscope kit (Advanced Cell Diagnostics, Hayward, CA, USA) with unstained tissue slides according to the manufacturer's instructions. Each tissue section underwent pretreatment involving heat and protease application before hybridization with a specific Axin2 probe. A horseradish peroxidase-based signal amplification system was hybridized to the probe, followed by color development using 3,3'-diaminobenzidine tetrahydrochloride. The housekeeping gene ubiquitin C was employed as a positive control, and the bacterial gene DapB served as a negative control. The patterns of brown punctate dots present in nucleus and/or cytoplasm interpreted positive staining. Axin2 expression was quantified according to the five-grade scoring system (grade 0: no staining, grade 1: 1-3 dots/cell, grade 2: 4-10 dots/cell, grade 3: > 10 dots/cell, grade 4: > 15 dots/cell with > 10% of dots in clusters) [13]. The grade and percentage of tumor cells expressing Axin2 were measured, and histoscores (H-scores) were calculated by multiplying the grade (range, 0 to 4) and percentage of Axin2-positive tumor cells (range, 1 to 100), ranging from 0 to 400. For statistical analysis, H-score of 68 was chosen as cutoff value based on the mean value of Axin2 H-score (H-score, 68); the tumor was defined as high when Axin2 H-score is higher than 68.

Statistical analysis

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) statistical software ver. 18.0 (SPSS Inc., Chicago, IL, USA) and Prism ver. 9.0.1 (GraphPad Software, San Diego, CA, USA). We analyzed the association of Axin2 with clinicopathological features with the Pearson χ^2 test. The correlations between the Axin2 H-scores and β -catenin expressions were evaluated by the Spearman correlation test. Difference was considered significant when $p < .05$.

RESULTS

Axin2 expression in the normal gastric mucosa with or without inflammation

To examine Axin2 expression in the non-tumorous stomach, we performed RNA in situ hybridization for Axin2 with 12 cases of normal gastric tissues (n = 12, comprising 20 spots): including samples from normal gastric mucosa without inflammation (5 spots), gastritis cases (5 spots), and IM (10 spots). We observed

a significant level of Axin2 expression in the normal stomach without inflammation. Axin2 was primarily expressed in the superficial epithelial cells and isthmus areas with an intensity level of 2 or lower (Fig. 1). In contrast, Axin2 expression was rarely observed at the gland bases. Then, we determined Axin2 expression in gastritis cases and observed a similar extent of Axin2 expression to as seen in the normal gastric mucosa (Fig. 2).

Axin2 expression in IM

IM is considered a precancerous lesion of GC. Thus, we evaluated Axin2 expression in IM and found that Axin2 expression was remarkably increased with an intensity level of 3 or higher both in the antrum and corpus. In particular, Axin2 expression was accentuated at the bases of IM glands (Fig. 3A), while the surface and isthmus regions displayed an intensity level of 2.

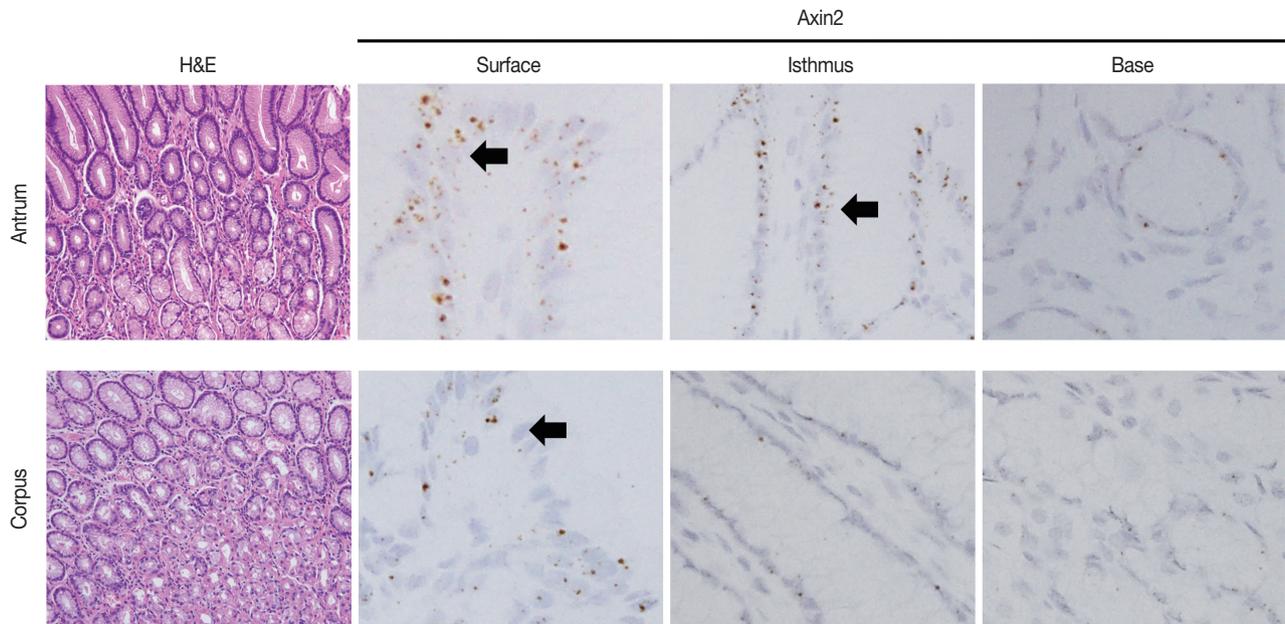


Fig. 1. Axin2 expression in normal gastric mucosa. Normal antrum and corpus show focal Axin2 expression (intensity level 2) in the superficial and isthmic areas.

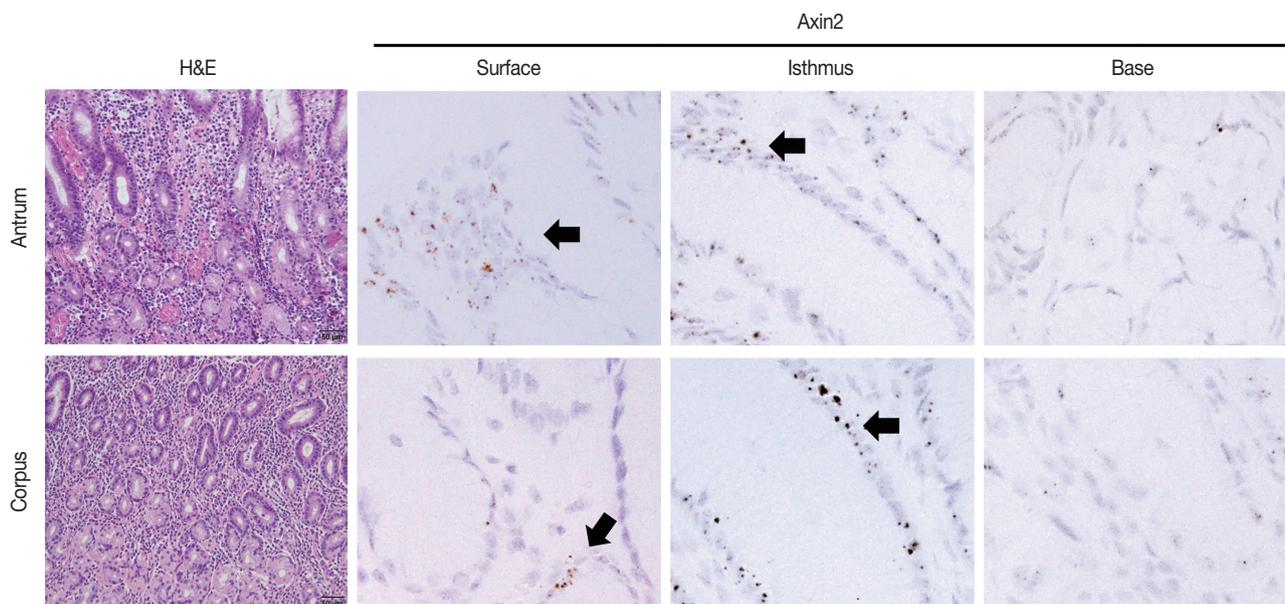


Fig. 2. Axin2 expression in gastric mucosa with chronic gastritis. Focal axin2 expression (intensity level 2) is observed in the superficial and isthmic areas of the antrum and corpus.

Likewise, goblet cells in the superficial area did not exhibit Axin2 expression, whereas those at the base region displayed an intensity level of 2 to 3. We determined H-scores of Axin2 in 12 non-tumorous tissue samples. Axin2 expression was significantly up-regulated in IM (H-score: mean \pm standard deviation [SD], 87.00 \pm 38.88) compared to normal (H-score: mean \pm SD, 18.00 \pm 4.47) and gastritis (H-score: mean \pm SD, 33.00 \pm 18.57) cases (Fig. 3B). These findings suggest that Axin2 expression might be associated intestinal differentiation or indicate a development into precancerous stage.

Associations of Axin2 expression with clinicopathological features in gastric carcinomas

Next, we examined Axin2 expression in 56 cases of early gas-

tric carcinomas and measured H-scores. Patient characteristics including age, gender, Lauren classification, histological differentiation, invasion depth, and lymph node metastasis is presented in Table 1. We considered Axin2 expression high when H-scores were higher than 68 based on the fact that mean value of H-score was 68. In total, 30% of GC cases showed high Axin2 expression (Table 1). No clinicopathological characteristic was found to be significantly associated with Axin2 expression (Table 1). Although it did not reach the statistical significance, high Axin2 expression was more often observed in GC patients with older age ($p = .062$), and intestinal-type gastric carcinomas tend to show high Axin2 expression ($p = .286$) (Fig. 4). Furthermore, lymph node metastasis was not observed in Axin2-high GC ($p = .108$).

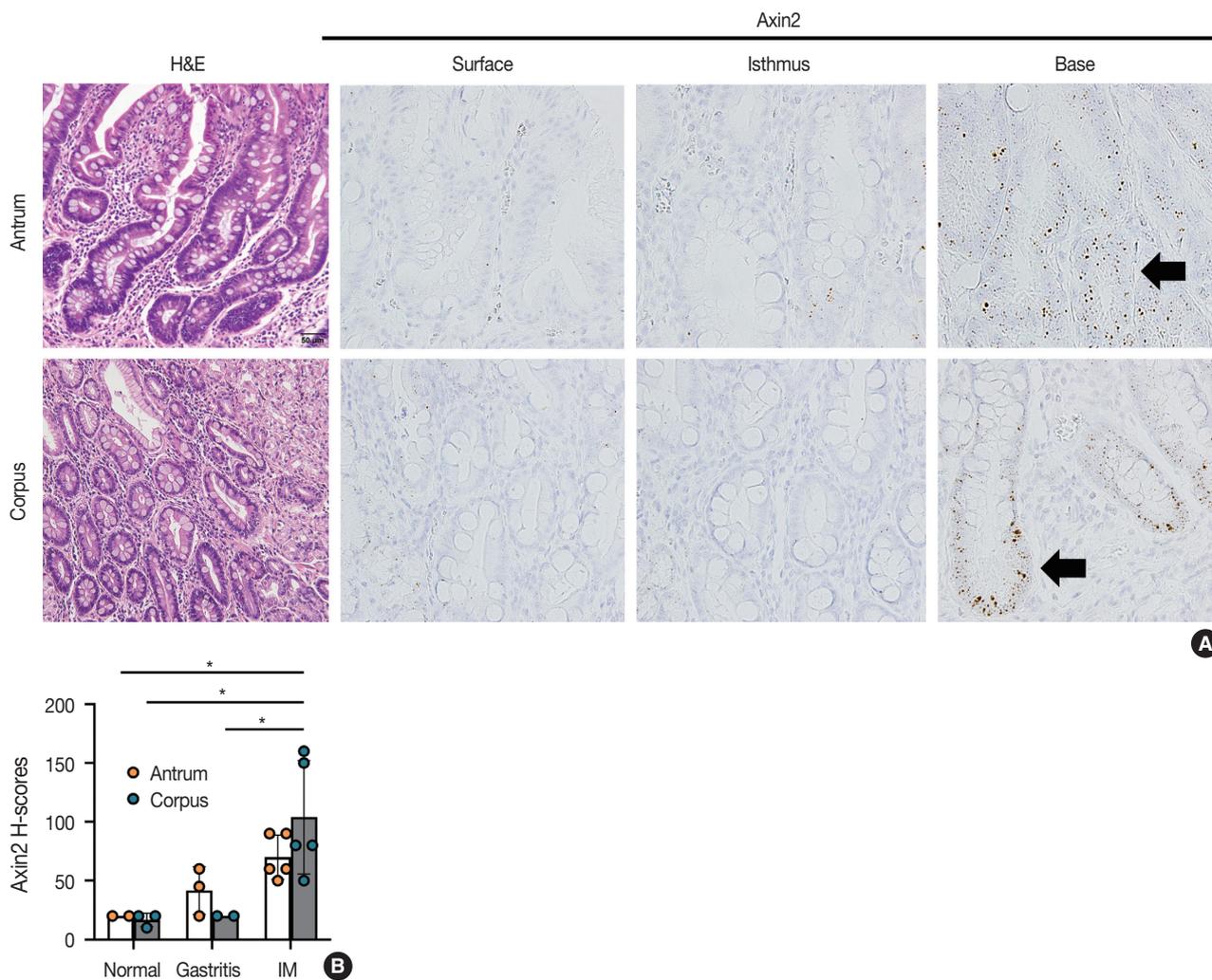


Fig. 3. Increased Axin2 expression in intestinal metaplasia (IM). (A) IM lesions in the antrum and corpus show enhanced Axin2 expression (intensity level 2–3) mainly at the bases. (B) A bar graph showing histo-scores (H-scores) of Axin2 in the non-cancerous stomach tissues, including normal gastric tissue, chronic gastritis, and IM cases. * $p < .05$.

Table 1. Association between Axin2 expression and clinicopathological characteristics in gastric carcinomas

| | Total | Axin2 | | p-value ^a |
|------------------------------|-----------|-----------|-----------|----------------------|
| | | Low | High | |
| No. of patients | 56 (100) | 39 (70.0) | 17 (30.0) | |
| Age (yr) | | | | .062 |
| <65 | 20 (35.7) | 17 (85.0) | 3 (15.0) | |
| ≥65 | 36 (64.3) | 22 (61.1) | 14 (38.9) | |
| Sex | | | | .449 |
| Female | 19 (33.9) | 27 (73.0) | 10 (27.0) | |
| Male | 37 (66.1) | 12 (63.2) | 7 (36.8) | |
| Lauren classification | | | | .286 |
| Intestinal | 46 (82.1) | 30 (65.2) | 16 (34.8) | |
| Diffuse | 8 (14.3) | 7 (87.5) | 1 (12.5) | |
| Mixed | 2 (3.6) | 2 (100) | 0 | |
| Histological differentiation | | | | .792 |
| Well | 11 (19.6) | 7 (63.6) | 4 (36.4) | |
| Moderate | 34 (60.7) | 23 (67.6) | 11 (32.4) | |
| Poor | 6 (10.7) | 5 (83.3) | 1 (16.7) | |
| PCC | 5 (8.9) | 4 (80.0) | 1 (20.0) | |
| Invasion depth | | | | .867 |
| Mucosa | 14 (25.0) | 10 (71.4) | 4 (28.6) | |
| Submucosa | 42 (75.0) | 29 (69.0) | 13 (31.0) | |
| LN metastasis | | | | .108 |
| Negative | 35 (85.4) | 24 (68.6) | 11 (31.4) | |
| Positive | 6 (14.6) | 6 (100) | 0 | |
| Nuclear β-catenin | | | | <.001 |
| Negative | 44 (78.6) | 37 (84.1) | 7 (15.9) | |
| Positive | 12 (21.4) | 2 (16.6) | 10 (83.4) | |

Values are presented as number (%).

PCC, poorly cohesive carcinoma; LN, lymph node.

^aPearson chi-square.

Nuclear β-catenin expression in gastric carcinomas and its correlation with Axin2

Nuclear β-catenin represents abnormal activation of Wnt/β-catenin pathway. Since Axin2 is one of the target genes of Wnt pathway, we hypothesized that Axin2 expression might be associated with nuclear β-catenin expression. Therefore, we performed IHC for β-catenin on GC cases and found that 12 cases (21%) of GC exhibited nuclear β-catenin expression (Table 1). Notably, Axin2 expression exhibited a strong positive association with nuclear β-catenin ($p < .001$) (Table 1). Representative images are presented in Fig. 5A. Among 44 cases with negative nuclear β-catenin, 37 cases (84%) displayed low Axin2 H-scores (mean \pm SD, 78.00 \pm 77.04), while seven cases (16%) exhibited high Axin2 H-scores. Conversely, in the subset of 12 cases featuring positive nuclear β-catenin, two cases (17%) demonstrated low Axin2 H-scores, while the remaining 10 cases (83%) exhibited high Axin2 H-scores (mean \pm SD, 179.40 \pm 87.09). Axin2 H-scores were significantly higher in GC with nuclear β-catenin

expression (Fig. 5B), clearly indicating that abnormally enhanced Wnt/β-catenin activity might be associated with elevated Axin2 expression.

DISCUSSION

When comparing the expression of Axin2 among normal, gastritis, and IM, normal and gastritis samples demonstrated H-scores below the average in the surface and isthmus regions, respectively. Conversely, IM predominantly exhibited elevated H-scores, particularly at the base of glands (Fig. 5). The absence of a substantial increase in Axin2 expression in gastritis suggests that mild to moderate gastritis does not induce activation of Wnt/β-catenin pathway. In contrast, Axin2 expression was significantly increased in cases of IM. We believe that this is the first study that has demonstrated the upregulation of Axin2 in IM. Considering that Axin2 is normally highly expressed in the crypt bases of normal intestines, Axin2 upregulation in IM seems to be associated with the transition into intestinal phenotype. This observation appears to align with previous research indicating that the progression of IM increases the susceptibility to dysplasia and carcinoma in the stomach [14]. However, further studies are required to fully understand the implications of Axin2 in IM, for example, whether it is involved in the progression of IM into dysplasia or gastric carcinoma. Despite the relatively limited number of IM cases in the present study, our results provided a connection between the IM and Axin2 expression.

Axin2 is a direct target gene of Wnt/β-catenin. It exerts control over the cytoplasmic levels of β-catenin by facilitating its degradation [1]. In the absence of Wnt signaling, the amino terminus of β-catenin undergoes phosphorylation by CK1 and GSK-3β, leading to its destruction through multi-protein destruction complex composed of APC, GSK-3β, Axin1, and conductin/Axin2. This process results in the proteasomal degradation of β-catenin [15,16]. In the nucleus, β-catenin bonds with TCF to activate expression of various genes including Axin2. Axin2 participates in the negative feedback regulation of Wnt/β-catenin signaling pathway by promoting the degradation of β-catenin [17]. Consistent with this role, Axin2 has been demonstrated to act as a tumor suppressor [16]. Carcinomas arising due to abnormalities in the Wnt/β-catenin signaling pathway exhibit increased expression of nuclear β-catenin [18]. Therefore, we suggest that increased Axin2 expression in gastric carcinomas is likely derived from the enhanced Wnt/β-catenin signaling pathway. Furthermore, it might be meaningful to explore the potential of high Axin2 expression as a prognostic factor for gastric car-

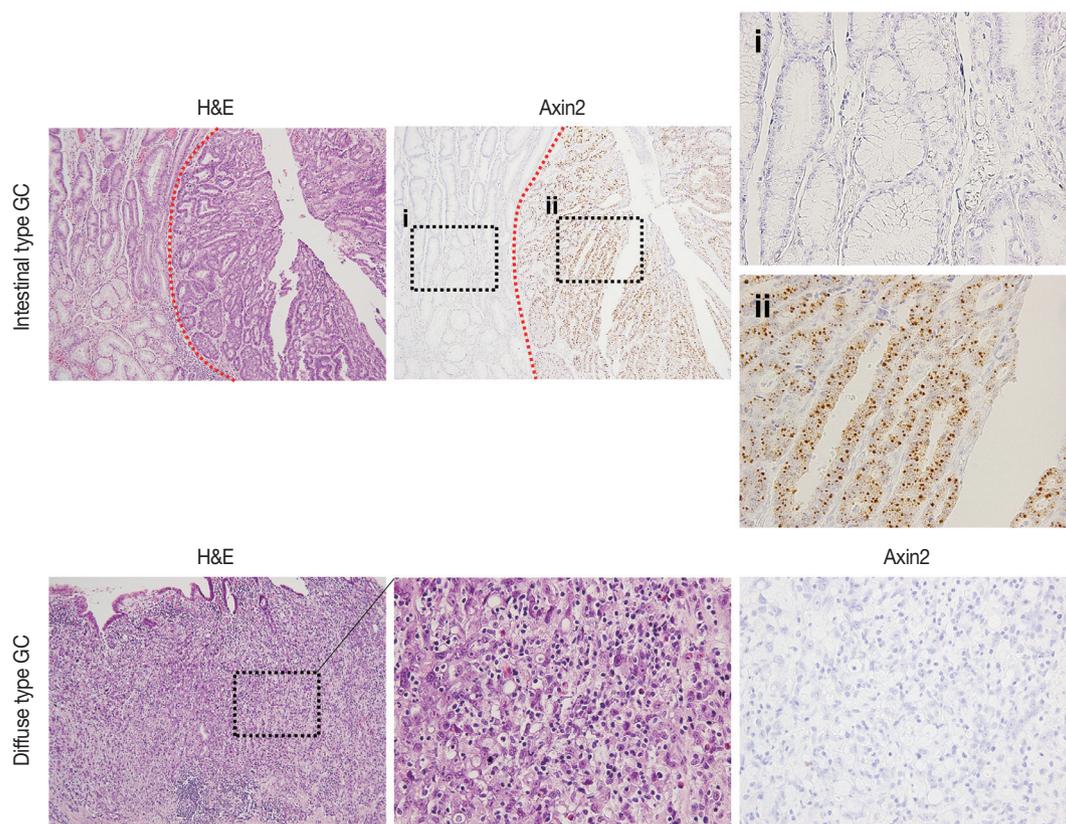


Fig. 4. Axin2 expression in gastric carcinomas according to Lauren classification. Representative intestinal (A) and diffuse type (B) gastric carcinomas showing high or low Axin2 expression. Red dotted lines indicate the border between normal gastric mucosa and gastric carcinoma. Black dotted boxes denote enlarged areas.

cinoma, similar to abnormal β -catenin nuclear expression [19].

One disappointing aspect of our findings is the absence of a discernible correlation between the expression level of Axin2 and the differentiation status of gastric carcinomas. This observation implies that the differentiation of GC might be governed by mechanisms independent of abnormal activation of Wnt/ β -catenin signaling pathway. As previously established, well-differentiated (WD) carcinoma and poorly differentiated (PD) carcinoma follow distinct carcinogenetic pathways [20]. While WD carcinoma is primarily associated with factors such as *H. pylori* infection, hTERT-positive stem cell hyperplasia, and genetic instability, PD carcinoma is linked to events like chromosome 17p's loss of heterozygosity (LOH), mutation or LOH of p53, and mutation or loss of E-cadherin [20]. Our initial hypothesis suggested that Axin2 might play a role in the differentiation process; however, our results did not yield substantial evidence to substantiate this notion.

This study has certain inherent limitations. Firstly, we did not simultaneously assess other components of the Canonical Wnt/ β -catenin signaling pathway. The progression of cancer cells can

occur through specific proteins other than β -catenin and Axin2. Further study is required to better understand the interactions between Axin2 and other essential proteins in Wnt signaling pathway. Secondly, there exists an imbalance in the number of cancer tissues exhibiting high Axin2 expression compared to low expression. Ideally, a more balanced distribution would be desirable. However, in our experimental setup, the ratio of patients with Axin2-high GC to those with Axin2-low GC was approximately 1:2. Consequently, future experiments with a greater number of Axin2-high GC could yield results differing from those obtained in this study. Furthermore, the evaluation of *Helicobacter pylori* infection and the noncanonical Wnt/ β -catenin signaling pathway was not included in our study. Prior research has reported an increase in nuclear β -catenin and TCF/LEF transactivation due to *H. pylori* [21,22]. Hence, exploring the potential association between *H. pylori* infection and Axin2's negative feedback could offer meaningful insights.

In summary, we discovered that Axin2 expression is significantly increased in IM compared to normal gastric tissue and gastritis, in line with the fact that IM is a precancerous stage for

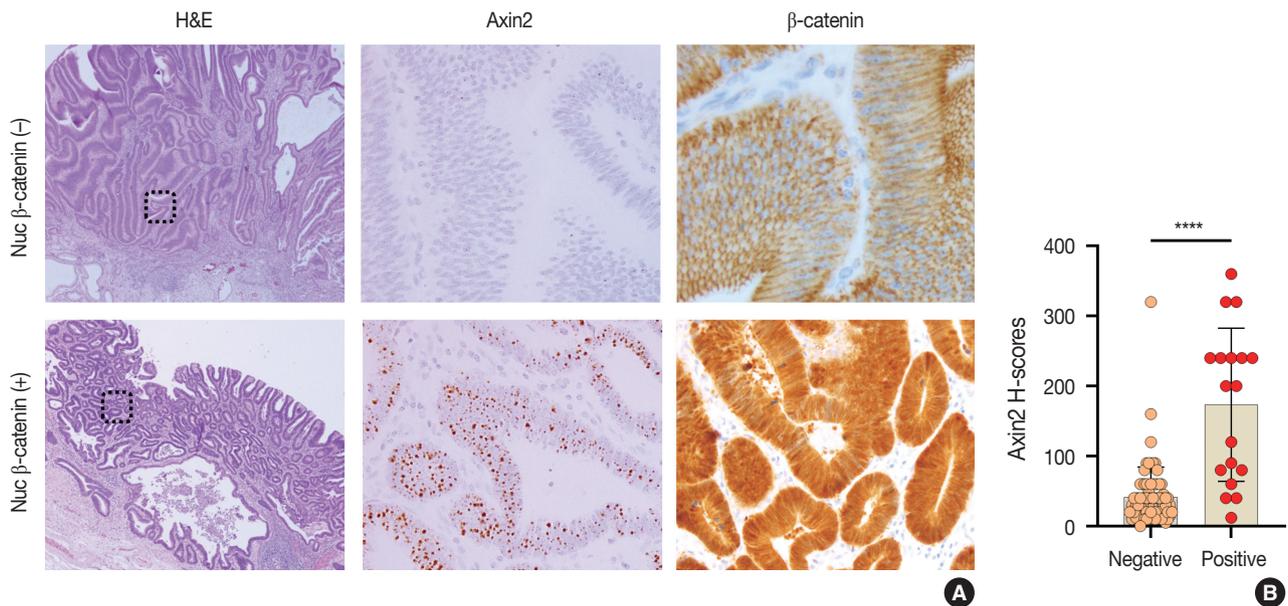


Fig. 5. Associations between Axin2 and nuclear β -catenin (nuc β -catenin) expression in gastric carcinomas. Representative cases showing low Axin2 and negative nuclear β -catenin expression (A) and high Axin2 and positive nuclear β -catenin expression (B) in gastric carcinomas. (C) A bar graph showing histo-scores (H-scores) of Axin2 in gastric carcinomas with negative nuclear β -catenin expression or positive nuclear β -catenin expression. **** $p < .0001$.

dysplasia or cancer progression. The expression level of Axin2 in GC was also associated with the nuclear expression of β -catenin, demonstrating the intimate relationship between Axin2 and Wnt/ β -catenin signaling pathway. Further research is required to explore the potential of Axin2 as a prognostic marker in GC.

Ethics Statement

This study was approved by the ethics committee of the Institutional Review Board of JNUH (IRB 2021-07-007) and was conducted in accordance with the Declaration of Helsinki. Informed consent from the patients was exempted by IRB approval.

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Code Availability

Not applicable.

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Author Contributions

Conceptualization: DHL, IHJ, BJ. Data curation: DHL, IHJ, BJ. Formal analysis: BJ. Investigation: DHL. Supervision: BJ. Visualization: DHL. Writing—original draft: DHL. Writing—review & editing: BJ. Approval of final manuscript: all authors.

Conflicts of Interest

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

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BRCA-mutated gastric adenocarcinomas are associated with chromosomal instability and responsiveness to platinum-based chemotherapy

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Background: Homologous recombination defect is an important biomarker of chemotherapy in certain tumor types, and the presence of pathogenic or likely pathogenic mutations involving *BRCA1* or *BRCA2* (p-BRCA) mutations is the most well-established marker for the homologous recombination defect. Gastric cancer, one of the most prevalent tumor types in Asia, also harbors p-BRCA mutations. **Methods:** To investigate the clinical significance of p-BRCA mutations, we analyzed 366 gastric cancer cases through next-generation sequencing. We determined the zygosity of p-BRCA mutations based on the calculated tumor purity through variant allelic fraction patterns and investigated whether the presence of p-BRCA mutations is associated with platinum-based chemotherapy and a certain molecular subtype. **Results:** Biallelic p-BRCA mutation was associated with better response to platinum-based chemotherapy than heterozygous p-BRCA mutation or wild type BRCA genes. The biallelic p-BRCA mutations was observed only in the chromosomal instability subtype, while all p-BRCA mutations were heterozygous in microsatellite instability subtype. **Conclusions:** In conclusion, patients with gastric cancer harboring biallelic p-BRCA mutations were associated with a good initial response to platinum-based chemotherapy and those tumors were exclusively chromosomal instability subtype. Further investigation for potential association with homologous recombination defect is warranted.

Key Words: Homologous recombination; Gastric neoplasms; Poly(ADP-ribose) polymerase inhibitors; Chromosomal instability; Genes, BRCA2

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Gastric cancer is the fifth most common cancer worldwide and the most common tumor in Korea [1,2]. Many patients with gastric cancer require chemotherapy, and platinum-based chemotherapy is one of the widely used regimens [3-7]. Although many combinations of cytotoxic chemotherapy have been developed, none of them could achieve satisfactory clinical outcomes. Thus, many molecularly targeted therapies have been tested; however, the efficacy of those drugs was quite limited in gastric cancer, except for anti-ERBB2 therapies [1-8].

The BRCA1 (BRCA) gene performs a homology-directed repair, which is one of the repair systems for double-strand DNA breaks [9]. Thus, pathogenic or likely pathogenic *BRCA1*

or *BRCA2* mutations (p-BRCA) lead to homologous recombination deficiency (HRD). Patients with ovarian cancers showing HRD respond to platinum-based chemotherapy or poly(ADP-ribose) polymerase (PARP) inhibitors through synthetic lethality action [10]. The association between HRD and responsiveness to platinum-based chemotherapy or PARP inhibitors has been reported in several tumor types, namely, ovary, prostate, pancreas, and breast cancers [11,12]. For other tumor types, the clinical significance of HRD is poorly understood.

Even if gastric cancer is the most common cancer among germline p-BRCA mutation carriers [13], the significance of germline p-BRCA mutations in this context is also poorly investigated.

Furthermore, the clinical significance of somatic p-BRCA mutations in gastric cancer is poorly understood, which is probably because the zygosity of p-BRCA mutations was not considered in most previous studies and gastric cancer is a heterogeneous disease in which four distinct molecular subtypes have been reported [12,14,15]. To solve this issue, we analyzed next-generation sequencing (NGS) data focusing on the presence of p-BRCA mutations and their zygosity in conjunction with the degree of chromosomal instability (CIN) and microsatellite instability (MSI) status. Then, we investigated whether the presence of p-BRCA mutations is associated with responsiveness to platinum-based chemotherapy in the context of molecular subtypes.

MATERIALS AND METHODS

Case selection and mutation analysis

Overall, 1,820 patients who were pathologically diagnosed with adenocarcinoma of the stomach and underwent clinical NGS of the tumor tissues between January 2017 and July 2022 were initially included. Among them, 65 patients with gastric adenocarcinoma harboring p-BRCA mutations were identified (Fig. 1). The functional effects of *BRCA1* or *BRCA2* mutations were predicted using the BRCA mutation database of the Department of Pathology, University of Utah (<https://arup.utah.edu/database/BRCA/>) (Supplementary Table S1). For *BRCA* wild-type cases, 301 of 1,751 patients with *BRCA* wild-type tumors were randomly selected. The MSI status was determined by our validated

NGS as described previously [16]. Ambiguous cases were confirmed by immunohistochemistry of MLH1, MSH2, MSH6, and PMS2. Finally, Epstein-Barr virus (EBV) in situ hybridization results were reviewed, when available.

NGS analysis

The NGS study was performed using our clinically validated OncoPanel AMC v4.3 panel, which is a DNA-based hybrid capture targeted gene panel using the NextSeq 550Dx Sequencing System (Illumina, San Diego, CA, USA) as described previously [16]. Briefly, this panel was approximately 1.2 Mbp with 33524 probes targeting 382 genes, including entire exons of 199 genes, 184 hot spots, and partial introns for eight genes often rearranged in cancer. For DNA extraction, the tumor area was macrodissected from formalin-fixed, paraffin-embedded tissue blocks to achieve > 20% tumor purity. Sequenced reads were processed and variants were called as described previously [17]; briefly, sequenced reads were processed by GATK (ver.4.2.6.1) pipeline, and variant calling was performed using VarDict (ver. 1.6). Common and germline variants from somatic variant candidates were filtered out with the common dbSNP build 141 (found in > 1% of samples), Exome Aggregation Consortium release 0.3.1 (<http://exac.broadinstitute.org>), and Korean Reference Genome database (<http://152.99.75.168/KRGDB>) and an in-house panel of normal variants except *BRCA1* and *BRCA2*.

Two pathologists manually reviewed NGS data to remove false positives or false negatives and curated functional effects of

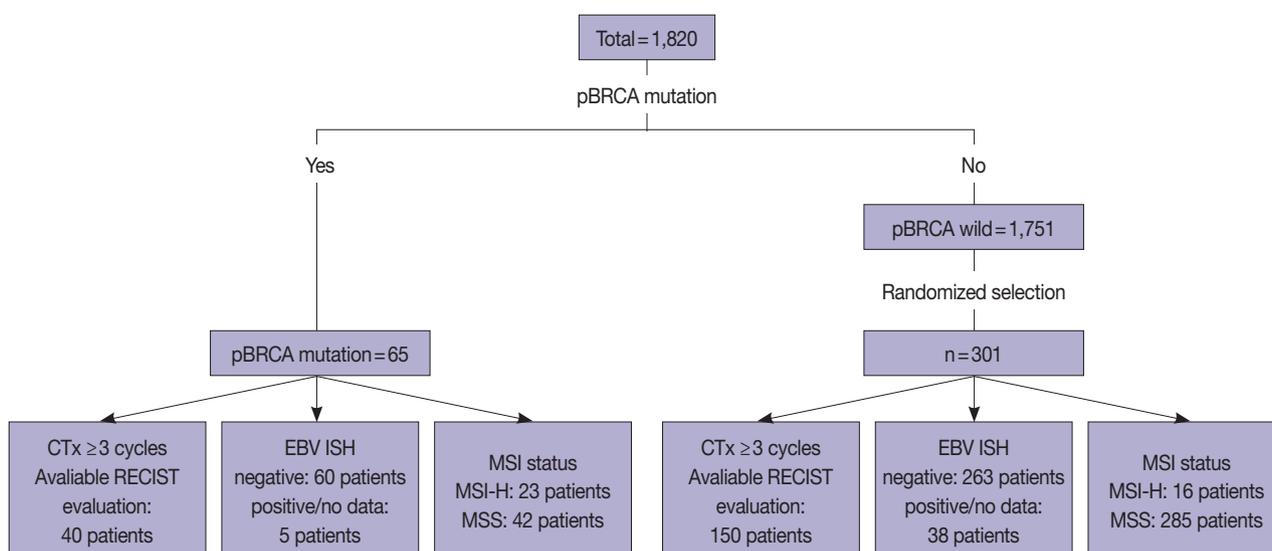


Fig. 1. Patient selection process. CTx, platinum-based chemotherapy; EBV ISH, Epstein-Barr virus in situ hybridization stain; MSI, microsatellite instability; MSI-H, high microsatellite instability; MSS, microsatellite stable; p-BRCA mutation, pathogenic or likely pathogenic *BRCA1* or *BRCA2* gene mutation; RECIST, modified Response Evaluation Criteria in Solid Tumors.

annotated *BRCA1* or *BRCA2* mutations. All detected single nucleotide variant and Indel types, including synonymous and non-synonymous mutations in all exonic regions and splice sites, were used to calculate tumor mutation burden (TMB) [18].

Copy number analysis

Copy number burden was calculated by the fraction of copy number altered regions across the human genome. The copy number altered regions were detected using CNVkit [19] with default parameters without normal tissue. Then, we classified tumors into CN-high (CIN) and CN-low (genomically stable) based on the relative copy number burden within EBV-negative/microsatellite stable (MSS) tumors. Briefly, we first sorted EBV-negative/MSS tumors in ascending order according to copy number burden, and then tumors with more than 25 percentile values were classified as CN-high tumors. This method was based on the previously published the Cancer Genome Atlas (TCGA) data where the frequency of the CN-high tumors was 72% while that of the CN-low (genomically stable) tumors was 28%, respectively, among the EBV-negative/MSS tumors [15].

Inference of the zygosity of p-BRCA mutations

Because *BRCA1* and *BRCA2* are tumor suppressors, complete loss of protein function might be biologically important. Thus, the zygosity of p-BRCA mutations or the loss of heterozygosity (LOH) of the p-BRCA-mutant allele in tumor tissues was necessary. To achieve this, tumor purity information was re-estimated through an analysis of variant allelic fraction (VAF) patterns of the detected variants rather than through pathologists' estimation, as described previously [20] with slight modification. Briefly, truncal mutations were carefully chosen. Truncal mutations are well-known oncogenes that are usually heterozygous. However, such oncogenes undergo LOH or mutant allele amplification on some occasions because of tumor-specific biology or selective pressure. Thus, we also considered local copy-number profiles and re-reviewed tumor slides from which DNA had been extracted.

As a simplified model, if the truncal mutation is heterozygous (or LOH-), tumor purity (T) would be two times of VAF (V): $T = 2V$. If the truncal mutation is biallelic (or LOH+), tumor purity (T) would be equal to VAF (V): $T = V$. Based on the calculated tumor purity, the zygosity of p-BRCA mutations could be determined.

If the reported VAF of the p-BRCA mutation was $T/2$, such a mutation was thought to be heterozygous (or LOH-). If it was the same as T, the p-BRCA mutation was thought to be biallelic

(or LOH+). If the reported VAF of the p-BRCA mutation was similar to $T+(1-T)/2$, the p-BRCA mutation was suggested to be a germline plus somatic LOH. The distinction between biallelic somatic and germline with or without LOH was not always possible. Thus, we grouped biallelic somatic and germline with or without LOH into the biallelic (or LOH+) category.

Evaluation of response to platinum-based chemotherapy

To evaluate responsiveness to platinum-based chemotherapy, we further selected 40 patients with *BRCA*-mutated gastric cancer and 150 with *BRCA* wild-type gastric cancer who underwent more than 3 cycles of platinum-based palliative chemotherapy and had valid response evaluation data (Fig. 1). Response to platinum-based chemotherapy was evaluated according to the modified Response Evaluation Criteria in Solid Tumors (RECIST ver. 1.1) [21]. When multiple combinations of chemotherapy were given, the initial response to the first platinum-based chemotherapy was used.

Statistics

Chi-square test and Wilcoxon signed-rank test were performed for pairwise comparisons. For nonparametric analysis between groups and variants, the Kruskal-Wallis test was used. The Kaplan-Meier method was performed for survival analyses, and the Mantel-Cox log-rank test was used to evaluate whether certain factors affect survival. p-values of $< .05$ were considered statistically significant. All statistical analyses and data visualizations were performed using IBM SPSS Statistics ver. 28.0.1 (IBM Corp., Armonk, NY, USA) and R-Studio using R ver. 4.2.2 (RStudio, Boston, MA, USA).

RESULTS

The presence of p-BRCA mutation is not associated with platinum chemotherapy response in gastric carcinoma when LOH of the p-BRCA mutant allele is not taken into consideration

Most clinicopathological variables for chemotherapy response were comparable between p-BRCA-mutant cases and *BRCA* wild-type cases (Table 1). Owing to the low prevalence of p-BRCA mutations, the finally selected patients were heterogeneous in terms of initial staging, treatment, and follow-up method. When zygosity of the p-BRCA mutations was not taken into consideration, the presence of p-BRCA mutations was not associated with responsiveness to platinum-based chemotherapy ($p = .833$) (Supplementary Table S2). In addition, overall survival

Table 1. Clinicopathological features of 366 patients with gastric cancer and complete NGS data

| Variable | p-BRCA mutation | | p-value |
|--|-----------------|---------------|---------|
| | Yes (n=65) | No (n=301) | |
| Age (mean) | 61.3 | 58.5 | .213 |
| Sex | | | .047 |
| Male | 51 (78.3) | 198 (65.7) | |
| Female | 14 (21.7) | 103 (34.3) | |
| Initial stage | | | .401 |
| I | 8 (11.6) | 13 (4.4) | |
| II | 5 (8.7) | 33 (10.8) | |
| III | 14 (23.2) | 82 (26.9) | |
| IV | 38 (56.5) | 173 (57.9) | |
| ERBB2 IHC positivity | 2 (3.1) | 40 (13.3) | .019 |
| Resectable at the time of diagnosis | 27 (43.5) | 129 (42.4) | .845 |
| Recurrence/metastasis after surgery | 16 (30.3) | 97 (32.7) | .228 |
| Histologic diagnosis | | | .076 |
| Adenocarcinoma, NOS | 52 (80.0) | 240 (79.7) | |
| Signet ring cell/poorly cohesive carcinoma | 6 (9.2) | 50 (16.6) | |
| Mucinous adenocarcinoma | 1 (1.5) | 4 (1.3) | |
| Medullary carcinoma with lymphoid stroma | 2 (3.1) | 3 (1.0) | |
| Other special variants | 4 (6.2) | 4 (1.3) | |
| Lauren classification | | | .114 |
| Intestinal | 20 (30.8) | 93 (30.9) | |
| Diffuse | 12 (18.5) | 68 (22.6) | |
| Mixed | 2 (3.1) | 62 (20.6) | |
| Indeterminate | 31 (47.7) | 78 (25.9) | |

Values are presented as number (%) unless otherwise indicated. NGS, next-generation sequencing; p-BRCA mutation, pathogenic or likely pathogenic *BRCA1* or *BRCA2* gene mutation; IHC, immunohistochemistry; NOS, not otherwise specified.

and progression-free survival were not affected by the presence of p-BRCA mutations (log-rank test, $p = .341$, and $p = .596$, respectively) (Fig. 2).

LOH of the p-BRCA mutant allele is an important predictor of responsiveness to platinum-based chemotherapy in gastric carcinoma

We hypothesized that biallelic p-BRCA mutations may result in the complete loss of BRCA protein function and hence may show a stronger association with responsiveness to platinum-based chemotherapy. Indeed, patients with gastric cancer harboring p-BRCA mutations accompanied by LOH showed a clear positive correlation with better responsiveness to platinum-based chemotherapy (relative risk, 1.74; 95% confidence interval, 1.136 to 1.531; $p = .001$) (Table 2). Notably, all gastric cancers showing p-BRCA mutations plus LOH showed objective responses. On multivariate analysis, MSI status, EBV in situ hybridization positivity, ERBB2 status, age, and sex were not associated with

platinum-based chemotherapy (data not shown). In contrast to the initial response to platinum-based chemotherapy, overall and progression-free survivals were not different according to the LOH of the p-BRCA mutant allele (Fig. 2).

The simultaneous p-BRCA mutation and LOH of the mutant allele is associated with increased copy number burden and CIN molecular subtype

Based on the association with responsiveness to platinum-based chemotherapy, we hypothesized that gastric cancers harboring p-BRCA mutation and LOH of the mutant allele might be associated with HRD and might be a good candidate for PARP inhibitor therapy. Approximately 40% of the p-BRCA mutations were accompanied by the LOH (Fig. 3A). Positions of observed mutations were scattered throughout the entire exonic area without any striking hotspot as usual tumor suppressors (Fig. 3B). VAFs were significantly higher in LOH+ group as expected (Fig. 3C). Genomic landscape of the included patients was generally consistent with the previously published large scale sequencing studies with *TP53* mutations being most frequent followed by *ARID1A* and *CDH1* mutations (Fig. 4A) [15,22]. The relatively frequent *BRCA2* mutations were due to selection bias related to study design and *BRCA2* mutations were far more frequent than *BRCA1* mutations. Interestingly, all p-BRCA mutations, that were accompanied by LOH, were found in MSS tumors, while the vast majority of the p-BRCA mutations without LOH were in MSI-high (MSI-H) tumors ($p < .001$) (Fig. 4B). Genetic alterations involving homologous recombination (HR)-related genes other than the *BRCA1* and *BRCA2* were observed in 117 samples (32%) but they did not show mutual exclusivity from those of *BRCA1* and *BRCA2* alterations (Supplementary Fig. S1).

Next, we focused on the copy number burden, namely, relative genomic regions showing copy number alterations of both loss and gain because it may serve as a surrogate for HRD score. Indeed, gastric cancers harboring p-BRCA mutations showed a higher copy number burden than those without p-BRCA mutations ($p = .029$) (Fig. 5A). Furthermore, within p-BRCA mutated tumors, tumors with concomitant LOH of the mutant allele were associated with a higher copy number burden than those without LOH ($p = .041$) (Fig. 5A). Then, when we classified cases with all required data, such as MSI status, EBV in situ hybridization result, and relative copy number burden, into the previously published four molecular subtypes, all gastric cancers harboring p-BRCA mutations with LOH belonged to CIN subtype while those harboring p-BRCA mutations without LOH were highly enriched in MSI-H subtype (Table 3) [15]. For TMB,

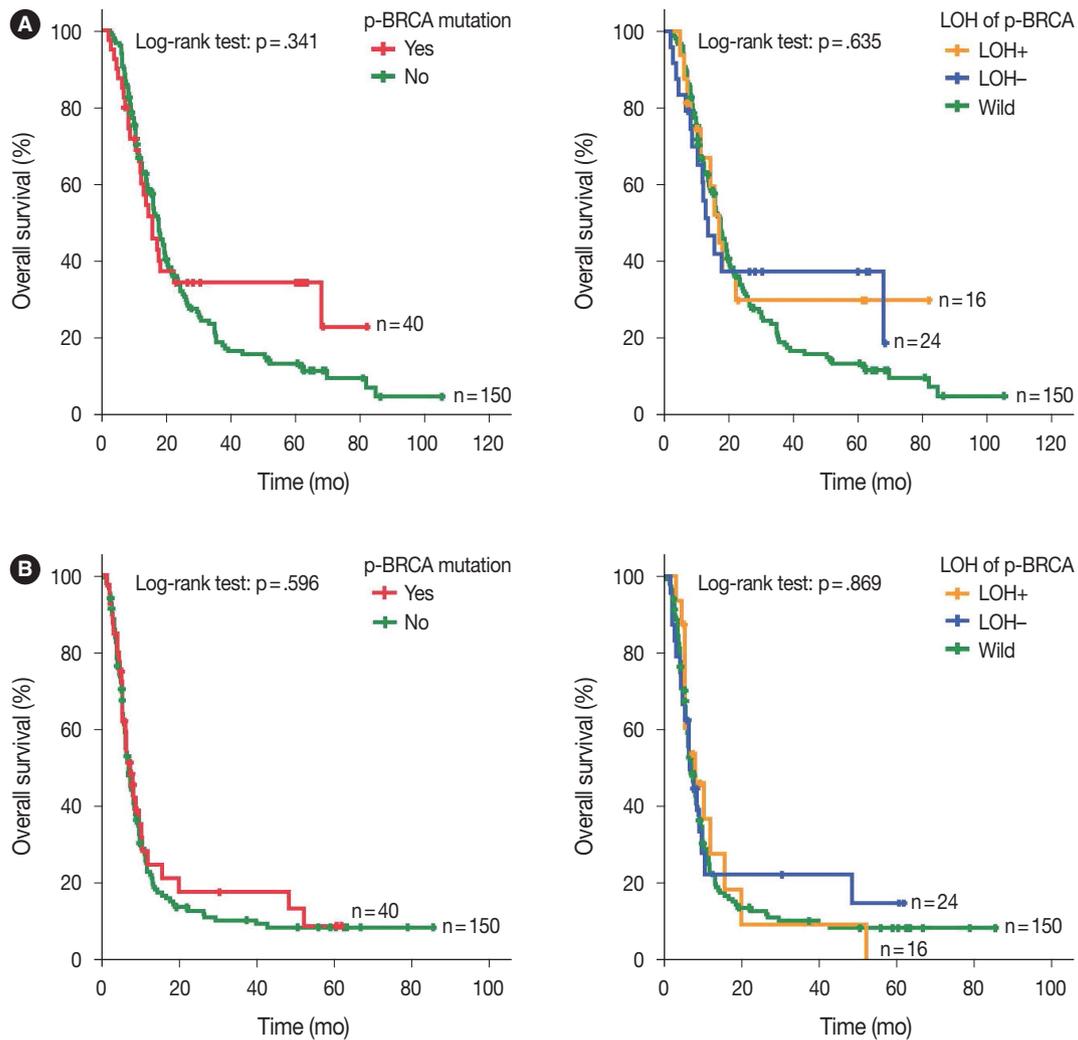


Fig. 2. Survival rate according to the p-BRCA mutation status. (A) The presence of p-BRCA mutations is not associated with overall survival regardless of LOH of the mutant allele. (B) The presence of p-BRCA mutations is not associated with progression-free survival regardless of LOH of the mutant allele. LOH⁻, no loss of heterozygosity of p-BRCA mutation; LOH⁺, loss of heterozygosity of p-BRCA mutation; p-BRCA mutation, pathogenic or likely pathogenic *BRCA1* or *BRCA2* gene mutation.

MSI-H subtype showed a higher TMB as expected, and p-BRCA mutated tumors showed a trend toward higher TMB than non-mutated tumors within MSS tumors (Fig. 5B). The representative copy number plots were depicted in Fig. 5C.

DISCUSSION

In this study, we showed that gastric cancer harboring p-BRCA mutations and LOH of the mutant allele is highly enriched in CIN molecular subtype and is associated with platinum-based chemotherapy. Although we could not directly evaluate HRD score and responsiveness to PARP inhibition, our findings suggest that patients with gastric cancer harboring biallelic p-BRCA

mutations constitute a distinct, genetically defined subgroup that shows HRD phenotype and, hence might be a potential candidate for PARP inhibitor therapy. Considering the fact that molecularly targeted therapies are pretty limited in gastric cancer, our findings shed light on potential PARP inhibitor therapy for patients with gastric cancer harboring biallelic p-BRCA mutations. In addition, the absence of improvement in progression-free survival by platinum-based chemotherapy implies that the PARP inhibitor therapy would be tried in an adjuvant setting rather than initially metastatic disease like high-grade serous carcinoma.

In terms of selecting patients who would benefit from PARP inhibitor therapy, the most validated criterion is the presence of

p-BRCA mutations. However, it has not been clearly defined whether the p-BRCA mutations should be accompanied by the LOH of the mutant allele. We found that the biological significance of p-BRCA mutation differs depending on the general genomic context. Patients with gastric cancer harboring p-BRCA mutations and LOH of the mutant allele showed a clear positive correlation with responsiveness to platinum-based chemotherapy, while those with gastric cancer harboring heterozygous p-BRCA

Table 2. Response to platinum-based chemotherapy according to p-BRCA mutation in the tumor tissue

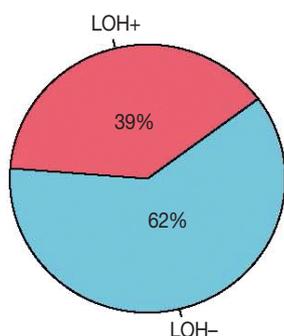
| Response | p-BRCA mutations and LOH status | | | p-value |
|---------------------|---------------------------------|-----------|-----------|-------------------|
| | LOH+ | LOH- | Wild | |
| Complete response | 1 (6.3) | 1 (4.2) | 5 (3.3) | .001 ^a |
| Partial response | 15 (93.8) | 8 (33.3) | 86 (57.3) | |
| Stable disease | 0 | 12 (50.0) | 52 (34.7) | |
| Progressive disease | 0 | 3 (12.5) | 7 (4.7) | |
| Total | 16 | 24 | 150 | |

Values are presented as number (%).
p-BRCA mutation, pathogenic or likely pathogenic *BRCA1* or *BRCA2* gene mutation; LOH, loss of heterozygosity.
^aKruskal-Wallis test.

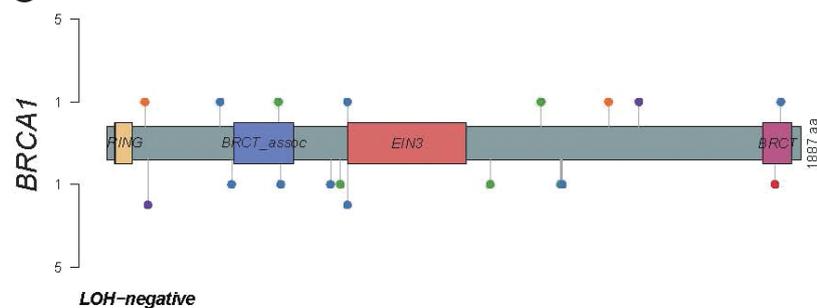
mutations were more like those with *BRCA* wild-type tumors. Furthermore, the vast majority of the heterozygous p-BRCA mutations were found in microsatellite-unstable tumors, and all p-BRCA mutations found in MSI-H gastric cancers were heterozygous. It can be explained by the biological characteristics of the MSI-H tumors that frameshift insertion or deletion mutations rapidly accumulate during tumor evolution, and those “passenger” p-BRCA mutations may hit quite large *BRCA1* or *BRCA2* genes [23]. As such, most p-BRCA mutations in MSI-H gastric cancers are just passengers that are not associated with the HRD phenotype. Finally, concomitant LOH of the p-BRCA mutant allele represents selection pressure toward HRD and may lead to complete loss of BRCA protein function and HRD phenotype. Indeed, the strong association between responsiveness to platinum-based chemotherapy and p-BRCA mutation plus LOH remained significant within the MSS subgroup ($p = .005$) (Supplementary Table S3).

Although inference of zygosity was done according to the methods described previously [20], it can be sometimes inaccurate because of inherent limitations of tumor-only sequencing.

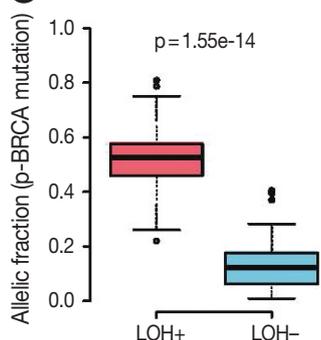
A Distribution of p-BRCA mutation (n=65)



B LOH-positive



C



LOH-positive

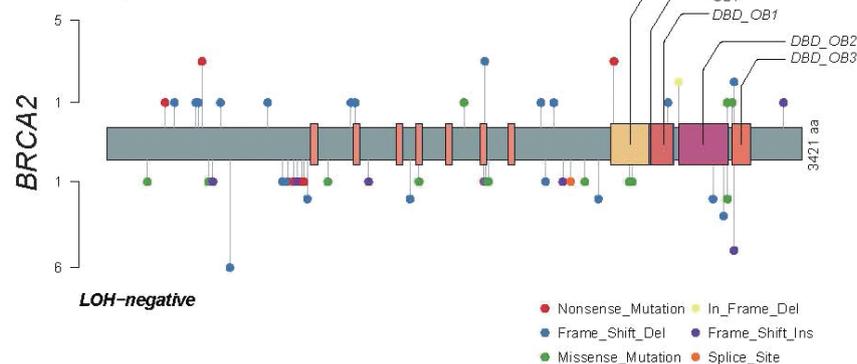


Fig. 3. Details of p-BRCA mutations. (A) Among the p-BRCA mutations detected in gastric cancers of 65 patients, 39% show loss of heterozygosity of the mutant allele (LOH+). (B) The detected p-BRCA mutations are scattered throughout the entire exonic area without major hotspots. (C) The variant allelic fractions of p-BRCA mutations with loss of heterozygosity are significantly higher than those of p-BRCA mutations without LOH ($p < .001$). p-BRCA mutation, pathogenic or likely pathogenic *BRCA1* or *BRCA2* gene mutation.

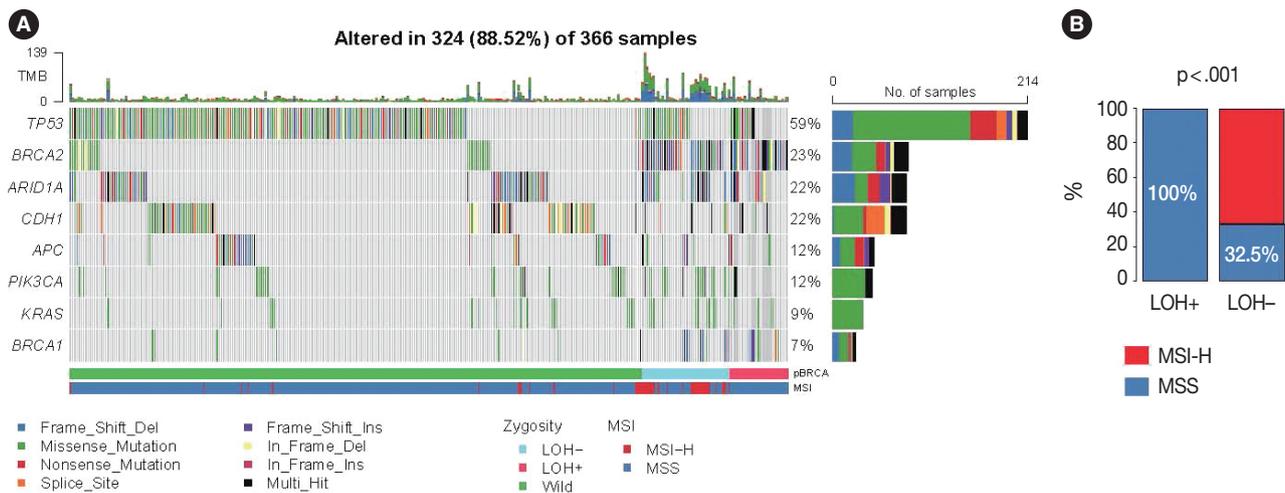


Fig. 4. Genomic landscape of selected gastric cancer samples. (A) The most frequently mutated genes include *TP53*, *ARID1A*, and *CDH1*. *BRCA2* mutations are far more frequent than *BRCA1* mutations. (B) All gastric cancers harboring p-BRCA mutations plus LOH of the mutant allele are MSS while most cases harboring heterozygous p-BRCA mutations are MSI-H. p-BRCA mutation, pathogenic or likely pathogenic *BRCA1* or *BRCA2* gene mutation; LOH, loss of heterozygosity; MSS, microsatellite stable; MSI-H, high microsatellite instability; TMB, tumor mutation burden.

Therefore, whole exome sequencing on matched tumor-normal pairs might be required for more accurate inference.

To further define molecular subgroups that can serve as a target population for HR-targeted therapies, we tried to classify our cases into the previously published four molecular subtypes [15] and found that gastric cancers harboring p-BRCA mutations plus LOH are exclusively CIN subtypes. Thus, we propose that potential targets for HR-targeted therapies should be screened in the CIN subtype of gastric cancers. During the assignment of molecular subtypes, we assumed that our study population is similar to that of the TCGA project rather than performing unsupervised clustering. We admit that our assumption may not be true because our sample set has a profound selection bias due to the case-control study design. External validation using independent sample sets with complete multi-omics and chemotherapy response data might be necessary to overcome this limitation. In addition, we could not directly measure HRD score for various reasons, and we used relative copy number burden as a surrogate marker. This may be a fundamental limitation because not all aneuploidies are associated with the HRD phenotype. Furthermore, we chose responsiveness to platinum-based chemotherapy as a clinical outcome because no patient has received PARP inhibitor therapy. To overcome those limitations, further studies involving direct measurement of HRD score or randomized clinical trials of PARP inhibitor therapies in genomically defined patient populations are needed.

Because our study population has a selection bias and potential confounders, the prognostic significance is hard to determine.

In a multivariate analysis including tumor stage, p-BRCA mutation, MSI status, ERBB2 status, and age as covariates, only tumor stage was independently associated with overall survival (data not shown). Notably, the p-BRCA mutation status plus LOH did not affect progression-free survival despite its association with good responses to platinum-based chemotherapy. Based on this observation, we propose that the efficacy of platinum-based chemotherapy in gastric cancer harboring biallelic p-BRCA mutations may be limited in the presence of measurable disease and that the potential use of PARP inhibition should be considered in an adjuvant setting after curative resection like high-grade serous carcinoma [24].

Because gastric carcinomas with p-BRCA mutation plus LOH are relatively infrequent, an efficient way of patient selection is needed. Our data suggest that potential candidates for PARP inhibitor therapy should be searched in the CIN subtype of gastric cancer because all gastric cancers harboring p-BRCA mutation and LOH of the mutant allele were exclusively found in the CIN subtype. However, identification of the CIN subtype is not easy in routine clinical practice. In this sense, the identification of histologic characteristics of those tumors might be helpful. Although we could not clearly define morphologic characteristics of gastric carcinomas with p-BRCA mutation plus LOH, those tumors tended to have an expansile tumor border, central necrosis, peri-tumoral Crohn-like lymphoid aggregates, and solid, cribriform, or papillary patterns (Supplementary Fig. S2). An artificial intelligence-based approach on a larger cohort may provide useful tools for the identification of those cases on a mor-

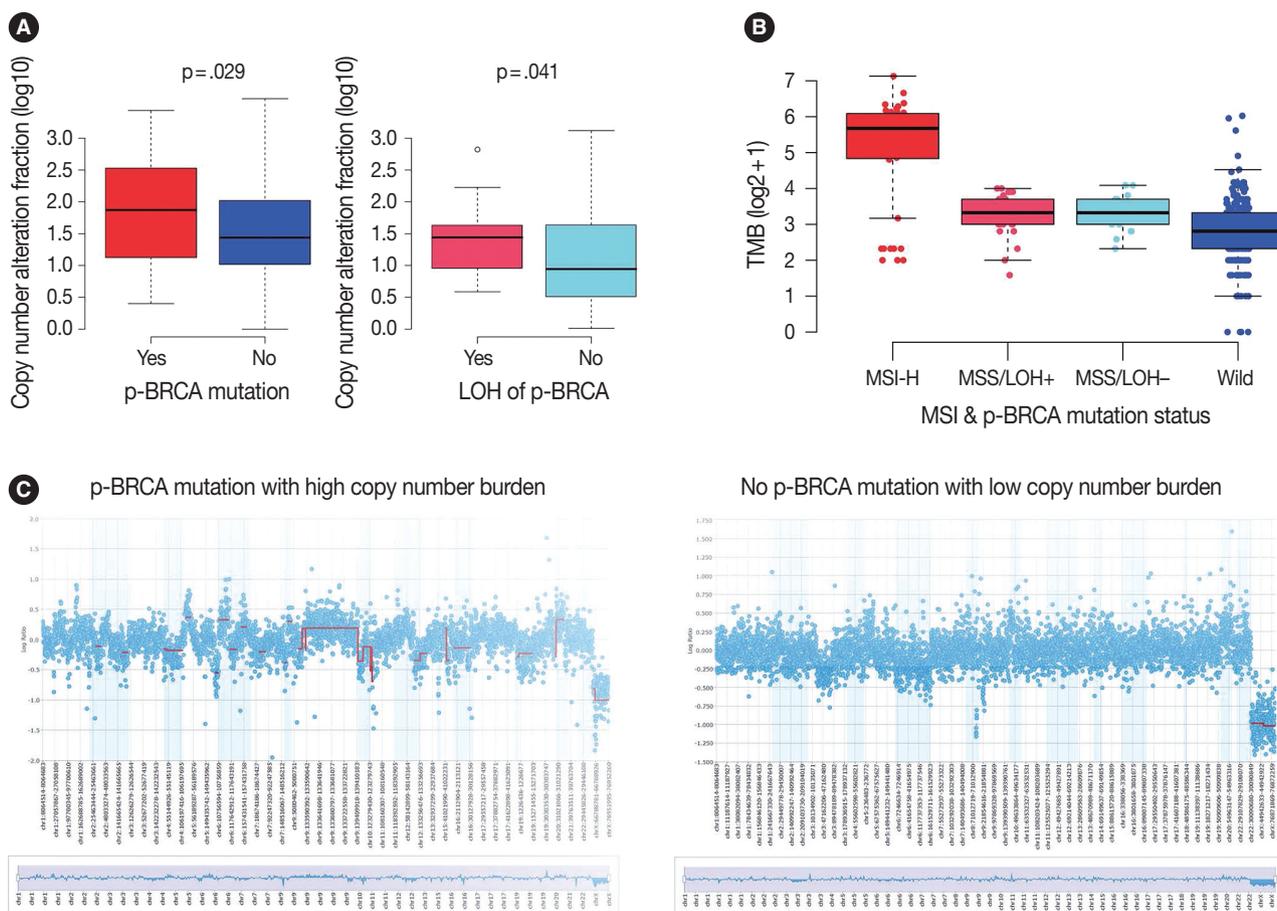


Fig. 5. Comparison of copy number and tumor mutation burden. (A) Copy number alteration fraction is significantly higher in p-BRCA mutated group than in *BRCA* wild-type group. Within p-BRCA mutated group, concomitant LOH is associated with a higher copy number burden. (B) Tumor mutation burden is different depending on p-BRCA mutation and MSI status (Kruskal-Wallis test, $p = 3.6 \times 10^{-12}$). Microsatellite-unstable tumors (MSI-H) are associated with high tumor mutation burdens. Within MSS subgroup, the p-BRCA mutated groups (MSS/LOH+ and MSS/LOH-) are associated with higher tumor mutation burdens than the *BRCA* wild-type group (Wild) ($p = .003$ each). The tumor mutation burden is not different according to LOH of the p-BRCA mutant allele ($p = .910$). (C) Representative copy number plots for tumors with high or low copy number burden are shown. Gastric cancer sample harboring a p-*BRCA* mutation and a high copy number burden (left) shows multiple segments with copy number gains or losses (indicated by red bars) while the *BRCA* wild-type tumor (right) does not. p-BRCA mutation, pathogenic or likely pathogenic *BRCA1* or *BRCA2* gene mutation; LOH, loss of heterozygosity; MSI-H, high microsatellite instability; MSS, microsatellite stable.

Table 3. Response to platinum-based chemotherapy according to p-BRCA mutation in the tumor tissue

| Response | p-BRCA mutations and LOH status | | | p-value |
|---------------------|---------------------------------|-----------|-----------|-------------------|
| | LOH+ | LOH- | Wild | |
| Complete response | 1 (6.3) | 1 (4.2) | 5 (3.3) | .001 ^a |
| Partial response | 15 (93.8) | 8 (33.3) | 86 (57.3) | |
| Stable disease | 0 | 12 (50.0) | 52 (34.7) | |
| Progressive disease | 0 | 3 (12.5) | 7 (4.7) | |
| Total | 16 | 24 | 150 | |

Values are presented as number (%). p-BRCA mutation, pathogenic or likely pathogenic *BRCA1* or *BRCA2* gene mutation; LOH, loss of heterozygosity. ^aKruskal-Wallis test.

phologic basis.

Taken together, patients with gastric cancer harboring p-BRCA mutations plus LOH of the mutant allele were associated with a good initial response to platinum-based chemotherapy, and such patients might be effectively identified by screening gastric cancers of CIN molecular subtype. Because responsiveness to platinum-based chemotherapy are known to be associated with HRD and responsiveness to PARP inhibitors, further investigations for HRD in gastric cancer tissue through validated methods and randomized clinical trials involving PARP inhibitors are warranted in the future.

Supplementary Information

The Data Supplement is available with this article at <https://doi.org/10.4132/jptm.2023.10.22>.

Ethics Statement

All procedures performed in the current study were approved by the Ethics Committee of Asan Medical Center, Seoul, Korea (IRB no. 2022-0956). Informed consent was waived by the Institutional Review Board.

Availability of Data and Material

Due to ethical limitations, the data analyzed in this study is not included in this manuscript. Under reasonable request, the corresponding author can provide the necessary information.

Code Availability

Not applicable.

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Conflicts of Interest

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

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Intravascular NK/T-cell lymphoma: a case report and literature review

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Intravascular lymphoma is characterized by an exclusively intravascular distribution of tumor cells. Intravascular natural killer/T-cell lymphoma (IVNKTL) is extremely rare, highly aggressive, commonly Epstein-Barr virus (EBV)-positive, and predominantly affects the skin and central nervous system. Here we report a case of IVNKTL diagnosed in a 67-year-old female, presenting with persistent intermittent fever and skin rashes throughout the body. Incisional biopsy of an erythematous lesion on the chest exhibited aggregation of medium to large-sized atypical lymphoid cells confined to the lumen of small vessels that were positive for CD3, granzyme B, and CD56 on immunohistochemistry and EBV-encoded RNA in situ hybridization. EBV DNA was also detected in serum after diagnosis. With a review of 26 cases of IVNKTL to date, we suggest that active biopsy based on EBV DNA detection may facilitate early diagnosis of IVNKTL.

Key Words: Malignant lymphoma; Extranodal NK-T-cell lymphoma; Epstein-Barr virus

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Intravascular lymphoma (IVL) is a rare extranodal lymphoma characterized by selective proliferation of lymphoma cells within the lumina of small blood vessels. IVL of the B-cell lineage is more common, and intravascular B-cell lymphoma was classified as a distinct disease entity in the revised 4th edition of the World Health Organization (WHO) classification of hematolymphoid tumors, while intravascular natural killer (NK)/T-cell lymphoma (IVNKTL) has not, and this has remained the same in the 5th edition [1,2]. IVNKTL is extremely rare and has been described as a rare extranodal NK/T-cell lymphoma (mass-forming) in the WHO classification. However, it differs from non-mass lesions based on distinct disease behavior. IVNKTL is mostly Epstein-Barr virus (EBV)-positive, highly aggressive, and predominantly affects the skin and central nervous system [3]. Since its pathological characteristics are still unclear, it was described as an aggressive NK-cell leukemia rather than an extranodal NK/T-cell lymphoma in the 5th WHO classification. However, further studies are required to determine where it fits best [2].

To the best of our knowledge, 26 cases of IVNKTL with sufficient immunohistochemical and clonality data have been re-

ported thus far, with two cases from Korea [4-23]. Herein, we report another case of IVNKTL with confirmed serum EBV DNA and review previously reported cases from a diagnostic perspective.

CASE REPORT

A 67-year-old woman with a history of asthma (adequately treated) was admitted with an intermittent night fever (39°C) lasting 1 month and general weakness that persisted after antibiotic treatment at a local hospital. Other vital signs were stable and there were no symptoms or signs other than fever, erythematous skin rash on the chest, and general weakness. Laboratory tests revealed slightly low white blood cell count, 3,090/mm³ (4000–10,000/mm³), and increased aspartate transaminase level of 99 U/L (0–37 U/L), alanine transaminase level of 42 U/L (0–41 U/L), and lactate dehydrogenase level of 2,600 U/L (135–225 U/L). Peripheral blood smear findings were normal. Immunological tests for antinuclear antibodies and rheumatoid factors were negative. Blood, cerebrospinal fluid (CSF), and urine cul-

tures also yielded negative results. Chest computed tomography (CT) revealed a focal consolidative lesion in the right lower lobe and bronchial wall thickening in both lungs. Slightly enlarged lymph nodes were found in the mediastinum and both hilar regions. Positron emission tomography showed multiple focal hot uptake lesions in bones, suggesting the differential diagnosis of multiple myeloma. However, a bone marrow biopsy and protein electrophoresis revealed no evidence of multiple myeloma or other malignancies. Abdominal and brain CT showed no remarkable findings. There was no history or evidence of hemophagocytic syndrome. The origin of the fever remained unclear. Given the recurrent fever, skin rash, and lymphadenopathy, adult-onset Still disease was suspected, and high-dose prednisolone was prescribed. After treatment, the fever subsided, but the skin rash showed little improvement. The patient developed a fever again, accompanied by multiple erythematous skin lesions all over her body. Her mental status decreased, and she underwent episodes of seizure. Brain CT and magnetic resonance imaging scans

showed no specific findings. An incisional skin biopsy of the anterior chest was performed.

Microscopic examination of skin specimen revealed mildly dilated capillaries in the dermis and superficial subcutaneous fat. Atypical lymphoid cells were confined to the lumen of the small blood vessels. The intravascular atypical cells were medium to large in size compared to normal lymphocytes and were poorly cohesive. The atypical cells had large, irregular nuclei and scanty cytoplasm (Fig. 1A). Occasional mitoses were observed. Immunohistochemistry (IHC) for CD31 highlighted the vasculature, and the intravascular presence of atypical cells was confirmed (Fig. 1B). Atypical cells were positive for CD3 (Fig. 1C), granzyme B (Fig. 1D), and EBV-encoded RNA (EBER) in situ hybridization (Fig. 1E). CD56 was weakly positive in the atypical cells (Fig. 1F). CD20, CD4, CD8, and CD30 expression were negative (Fig. 1G–J), and the Ki-67 proliferation index was >90% (Fig. 1K). A T-cell receptor (TCR) gene rearrangement test did not show monoclonal T-cell proliferation. In conclusion,

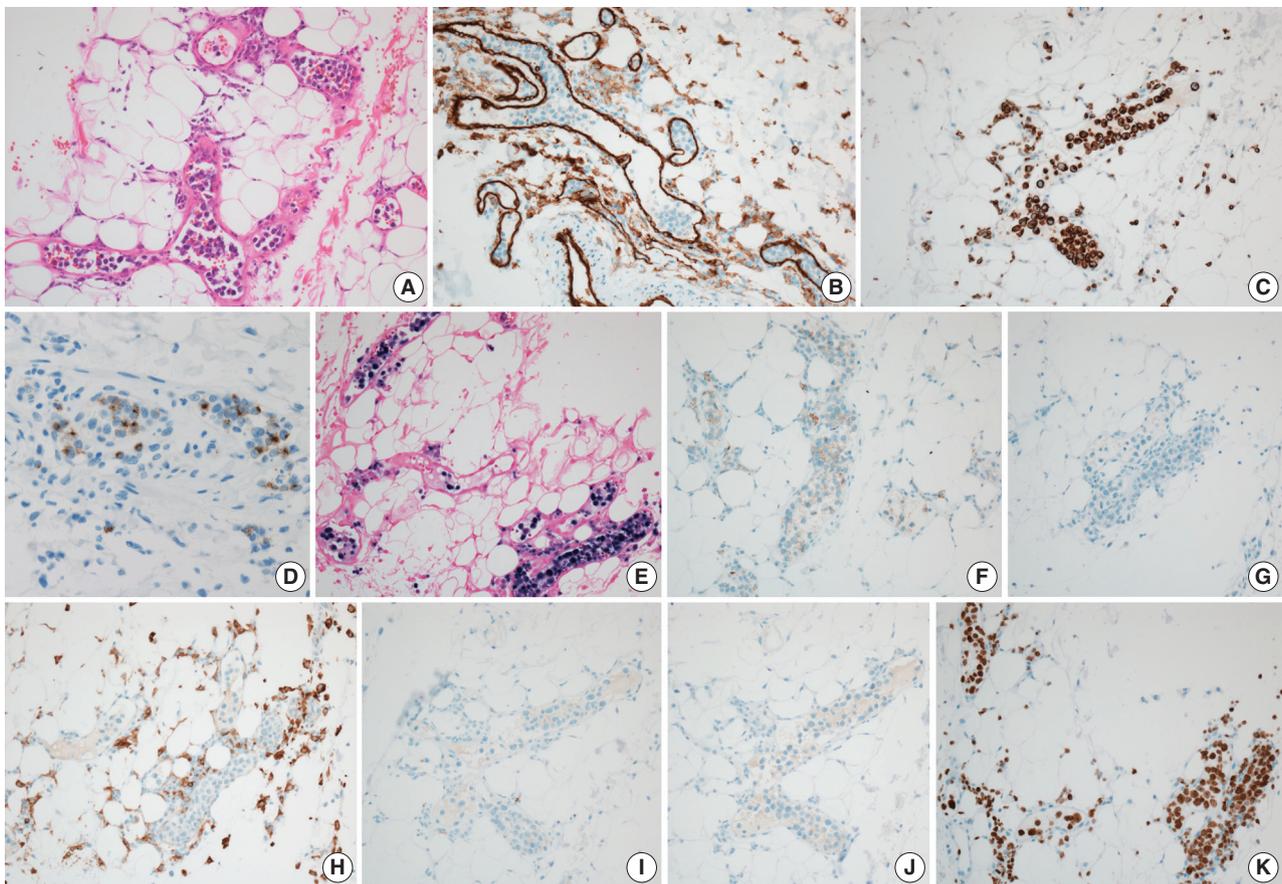


Fig. 1. Microscopic findings of cutaneous intravascular natural killer/T-cell lymphoma. Medium-sized atypical lymphoid cells can be seen in subcutaneous vessels (A). CD31 highlights vasculature (B), and atypical cells are positive for CD3 (C), granzyme B (D), and Epstein-Barr virus–encoded RNA in situ hybridization (E). CD56 shows weak positivity in atypical cells (F). CD20 (G), CD4 (H), CD8 (I), and CD30 (J) are negative. Ki-67 index is more than 90% (K).

Table 1. Summary of reported cases of intravascular NK/T-cell lymphoma

| Case | Age (yr)/ Sex | Biopsy sites | Skin lesion | CNS inv. | Cytopenia | Bone marrow inv. | EBV DNA | EBER | TCR | Survival |
|-----------------------------------|---------------|--------------|-------------|----------|-----------------------|------------------|---------|------|-------------------|---------------------------------|
| Santucci et al. (2003) [4] | 54/M | Skin | + | + | Leuko- | NA | NA | + | NA | Died 17 mo after dx. |
| Wu et al. (2005) [5] | 41/M | Skin | + | - | - | - | NA | + | Germline | Alive 12 mo after dx. |
| Wu et al. (2005) [5] | 47/F | Skin | + | + | Pan- | + | NA | - | Germline | Died 15 days after dx. |
| Kuo et al. (2006) [6] | 71/F | Skin | + | - | - | - | NA | + | Germline | Alive 5 mo after dx. |
| Song et al. (2007) [7] | 40/F | Skin | + | + | - | - | NA | + | Germline | Alive 7 mo after dx. |
| Nakamichi et al. (2008) [8] | 23/F | Skin | + | - | - | - | NA | + | Germline | Died 9 mo after dx |
| Cerroni et al. (2008) [9] | 67/F | Skin | + | + | - | NA | NA | NA | Clonal (TCRg) | Died 7 days after dx. |
| Cerroni et al. (2008) [9] | 63/M | Skin | + | - | Leuko-Thrombo- | NA | NA | + | Germline | Died 6 mo after dx. |
| Cerroni et al. (2008) [9] | 64/M | Skin | + | NA | - | - | NA | - | Germline | Died 7 mo after dx. |
| Cerroni et al. (2008) [9] | 87/M | Skin | + | - | - | NA | NA | + | Clonal (TCRg) | Died 15 days after dx. |
| Gleason et al. (2008) [10] | 62/M | Skin | + | - | - | - | NA | - | Clonal (TCRg) | Alive 8 mo after dx. |
| Liao (2011) [11] | 42/F | Skin | + | - | - | - | NA | + | NA | Alive 14 mo after dx. |
| Yanning et al. (2013) [12] | 84/F | Skin | + | NA | - | NA | NA | + | Germline | Alive 4 mo after dx. |
| Gbauer et al. (2014) [13] | 72/M | Skin | + | + | Pan- (6 mo after dx.) | + | NA | + | Germline | Died 7 mo after dx. |
| Jang et al. (2014) [14] | 23/F | Skin | + | - | - | - | NA | + | Germline | Died 18 mo after dx. |
| Liu et al. (2014) [15] | 38/F | Skin | + | - | - | - | NA | + | NA | Died 13 mo after dx. |
| Wang et al. (2015) [16] | 45/M | Skin | + | NA | - | NA | NA | + | Germline | Died 15 days after dx. |
| Wang et al. (2015) [16] | 32/F | Skin | + | - | - | NA | NA | + | Germline | Died 4 mo after dx. |
| Wang et al. (2015) [16] | 18/F | Skin | + | - | - | - | NA | + | Germline | Alive 3 yr after dx. |
| Bi et al. (2015) [17] | 29/M | Skin | + | - | - | - | NA | + | Germline | Died 3 mo after dx. |
| Alhumidi (2015) [18] | 48/F | Skin | + | - | - | - | NA | + | NA | Alive 18 mo after dx. |
| Okonkwo and Jaffe (2017) [19] | 51/M | Skin | + | + | - | NA | NA | + | Clonal (TCRg) | NA |
| Sharma et al. (2017) [20] | 62/F | Brain | - | + | - | + | NA | - | Clonal | Died 7 days after dx. |
| Alegria-Landra et al. (2017) [21] | 81/M | Skin | + | - | - | - | NA | + | Clonal (TCRb & g) | Died 15 days after dx. |
| Zanelli et al. (2018) [22] | 54/M | Autopsy | - | + | Pan- | + | + | + | Clonal | Died 18 days after presentation |
| Weisner et al. (2022) [23] | 53/M | Brain | - | + | - | - | + | + | NA | Alive 8 yr after SCT |
| Present case | 67/F | Skin | + | + | Leuko- | - | + | + | Germline | Died 18 days after dx. |

NK, natural killer; CNS, central nervous system; inv., involvement; EBV, Epstein-Barr virus; EBER, Epstein-Barr virus-encoded small RNA; TCR, T-cell receptor gene rearrangement; M, male; F, female; dx., diagnosis; NA, not available; IHC, immunohistochemistry; PE, pleural effusion; SCT, stem cell transplantation; sx., symptom.

the patient was diagnosed with cutaneous intravascular EBV-positive NK/T-cell lymphoma. After diagnosis, polymerase chain reaction (PCR) detected EBV DNA (445,000 copies/mL) in serum. The patient's clinical condition deteriorated with acute respiratory failure, and her mental status did not improve. She and her family opted for hospice care and no further pathological examination was done, but the progression of symptoms suggested that multiorgan failure and brain involvement had occurred. The patient expired 18 days after diagnosis.

DISCUSSION

IVNKTL is an extremely rare, highly aggressive lymphoma that predominantly involves the skin and central nervous system. Histological diagnosis is essential. Twenty-seven cases of IVNKTL, including the present case, with sufficient immunohistochemical and clonality data, have been reported (Table 1). The patients included 13 men and 14 women aged 18–87 years. The clinical presentation varied, including skin rash, neurologic symptoms, fever, cytopenia, weight loss, and malaise. Most patients had skin lesions (23/27, 85.2%, skin lesion only in 11 patients). Central nervous system involvement was found in 10 patients (10/27, 37.0%). Most of tested cases showed EBER positivity (22/26, 84.6%). Seven cases with clonality data showed the presence of monoclonal proliferation (7/22, 31.8%).

A skin biopsy is the easiest and most effective way to diagnose IVNKTL; however, it is challenging in patients without obvious skin lesions. Of the four patients without skin lesions, two presented with neurological symptoms and were diagnosed by brain biopsy (cases No. 23 and 26), and the other two were confirmed on autopsy (cases No. 3 and 25). Brain biopsy is more burdensome than skin biopsy. In each case, brain biopsy was performed after neurological symptoms worsened, and EBV DNA was detected in the CSF. The other two patients showed various symptoms and signs, including pancytopenia at presentation, and were diagnosed by bone marrow biopsy and autopsy. Autopsies of the last two patients revealed multiorgan involvement of IVNKTL, including the brain, kidneys, and bone marrow.

Bone marrow involvement was uncommon (4/19, 21.1%) and seemed to be associated with poor prognosis. In the case reported by Gebauer et al. [13] (case no. 14), bone marrow biopsy revealed dense medullary infiltration of IVNKTL, constituting approximately 40% of the overall cellularity. However, bone marrow biopsies showed only subtle sinusoidal lymphomatous infiltration in the other two cases (cases Nos. 3 and 25). The remaining case exhibited no evidence of neoplastic infiltration in morphologi-

cal and IHC analyses of the bone marrow biopsy, and only TCR gene rearrangement test of the bone marrow aspirate confirmed clonality (case No. 23). Although bone marrow involvement may be uncommon, it can also easily be missed. Meanwhile, patients with bone marrow involvement showed shorter survival (<1 month) after confirmation of bone marrow involvement. Bone marrow involvement may indicate rapid disease progression; however, further evaluation is needed.

Most reports did not mention EBV DNA PCR testing (24/27, 88.9%). In three recent cases, EBV DNA PCR of pleural effusion, lung tissue (case No. 25), CSF (case No. 26), and blood (present case) was positive. Serum EBV DNA load has been suggested as a biomarker in EBV-associated cancers such as nasopharyngeal carcinoma, Burkitt lymphoma, and EBV-positive Hodgkin lymphoma [24]. IVNKTL may also present with a high serum EBV DNA load. Since procedures for histopathological examination (e.g., brain biopsy) are invasive, detection of serum EBV DNA could provide a supportive basis for conducting further work-up for diagnosis, especially in patients without apparent skin lesions.

In conclusion, we reported a case of IVNKTL in which serum EBV DNA was detected. An active skin biopsy or other invasive biopsy based on EBV DNA detection may facilitate early diagnosis of IVNKTL.

Ethics Statement

This study was approved by the Institutional Review Board of Gyeongsang National University Hospital, and the need for informed consent was waived (IRB No. GNUH 2023-07-033).

Availability of Data and Material

The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Code Availability

Not applicable.

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Conflicts of Interest

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

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What's new in dermatopathology 2023: WHO 5th edition updates

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Abstract

The 5th edition WHO Classification of Skin Tumors (2022) has introduced changes to nomenclature and diagnostics. Important differences are discussed below. Changes in each category of skin tumor have been detailed, with particular emphasis on meaningful advances in our understanding of the molecular pathogenesis of the skin's diverse tumor landscape.

KERATINOCYTIC/ EPIDERMAL TUMORS

- Keratoacanthoma is kept separate from squamous cell carcinoma (SCC). Continued recognition that keratoacanthoma likely represents an SCC variant with self-resolving potential (Fig. 1).
- Adenosquamous carcinoma has been removed as an SCC subtype. It is thought to represent squamoid eccrine ductal carcinoma; discussed with adnexal neoplasms.

- Merkel cell carcinoma (Fig. 2) designated a neuroendocrine carcinoma of skin. In non-Merkel cell polyomavirus (MCPyV)-associated cases, *TP53* and *RB1* mutations seen in other neuroendocrine carcinomas are identified. Origin cell remains unclear.

MELANOCYTIC NEOPLASMS

- Advanced understanding of the mo-

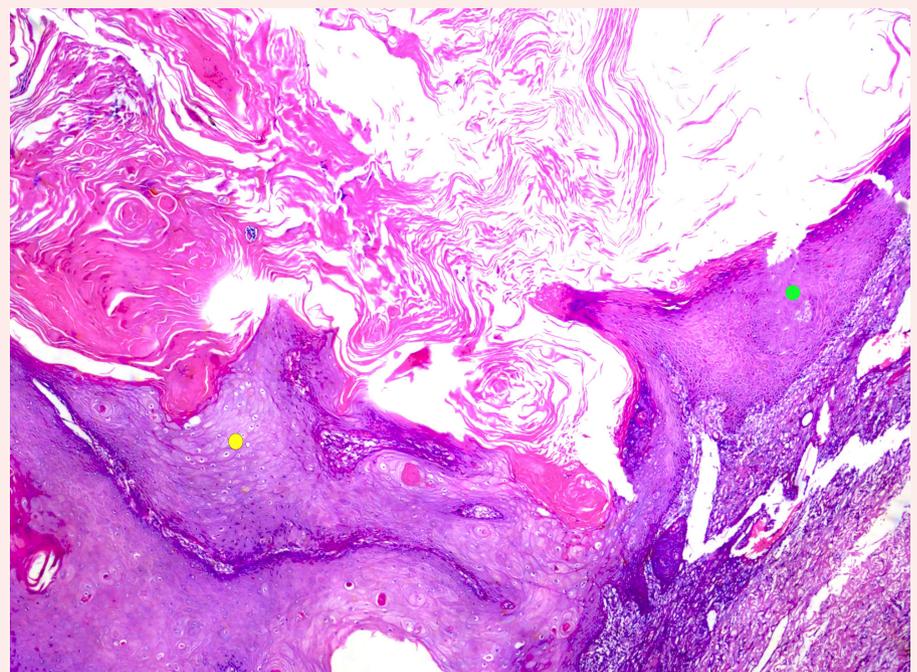


Fig. 1. Keratoacanthoma. This crateriform squamous cell carcinoma variant possesses self-resolving potential. Thick glassy epithelium (yellow dot) may regress via thinner basophilic epithelium (green dot) as shown in the image.

lecular pathways in melanocytic lesions have led to definition changes. Re-restrained proliferation of activated oncogenes by tumor suppressor genes leads to nevi, while additional mutations and escape from tumor suppressor gene control result in intermediate/malignant neoplasms [1].

- **Nevi:** clonal neoplasms with a single mutation, no other pathogenic changes, bland cytologic appearance and benign behavior.
- **Melanocytomas:** intermediate neoplasms between nevi and melanomas harboring > 1 driver mutation. Display atypical histopathologic features and

potential for local recurrence. Second mutations affect specific pathways resulting in reproducible clinical and microscopic features.

- Some melanocytomas (previously designated “nevi”) have been renamed to reflect respective specific pathway aberrations and their intermediate status.

- Wnt-activated deep penetrating/plexiform melanocytoma (Fig. 3)
- Pigmented epithelioid melanocytoma (PEM, also known as *PRKARIA*-inactivated melanocytoma)
- *BAP1*-inactivated melanocytoma
- Spitz melanocytoma (previously atypical Spitz tumor)
- Microphthalmia-associated transcription factor (*MITF*) pathway-activated melanocytic tumors, see below

- Immunohistochemistry can aid in diagnosis:

- Loss of expression of PrkAr1a in some PEM
- Diffuse nuclear β -catenin, nuclear LEF1 in Wnt-activated deep penetrating/plexiform melanocytoma [2].
- Loss of nuclear BAP1 expression in *BAP1*-inactivated melanocytoma

- *MITF* pathway-activated melanocytic tumors are a newly introduced set of melanocytomas with cytoplasmic clearing and fusion genes resulting in overactive *MITF* functioning [3,4]. Main differentials include clear cell sarcoma, PEComa, melanoma and carcinomas. Two variants are described

- Clear cell tumor with melanocytic differentiation and *ACTIN::MITF* Translocation (CCTMAM)
 - Cutaneous nodule
 - Dermal based +/- subcutis. Marked cytoplasmic clearing. Low/high-grade nuclear features but mitoses are inconspicuous and the lesion lacks ulceration or perineural invasion. MART-1, HMB45, S100, *MITF* positive. Pankeratin negative.
- Clear cell tumor with melanocytic differentiation and *MITF::CREM* translocation (CCTMMC)
 - Cutaneous nodule
 - Dermal based +/- subcutis
 - Marked cytoplasmic clearing (high-

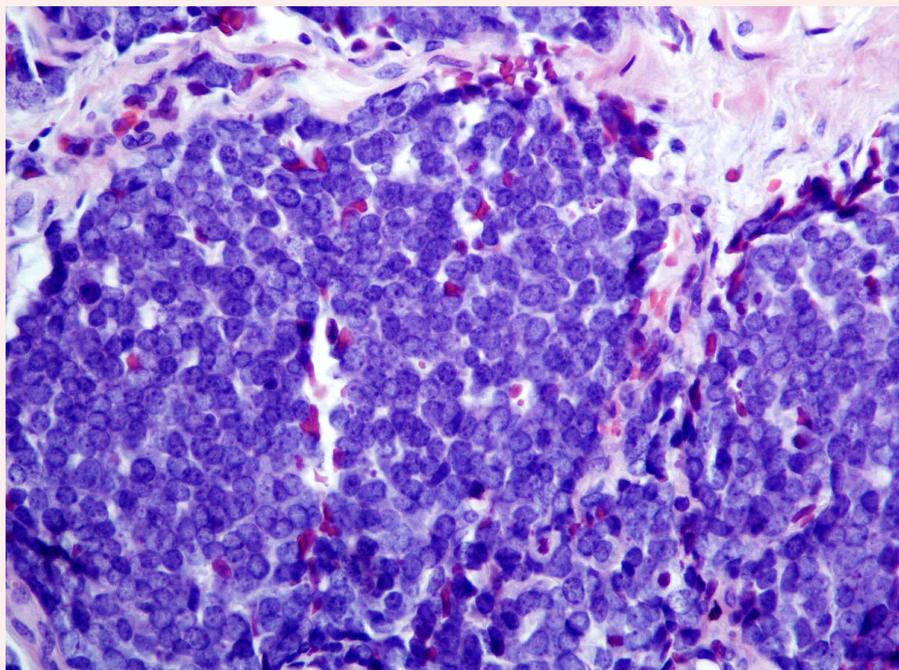


Fig. 2. Merkel cell carcinoma (primary cutaneous neuroendocrine carcinoma of skin). The tumor demonstrates round blue cells with a characteristic finely dispersed “salt and pepper” chromatin pattern. Both Merkel cell polyomavirus associated and non-associated cases exist.

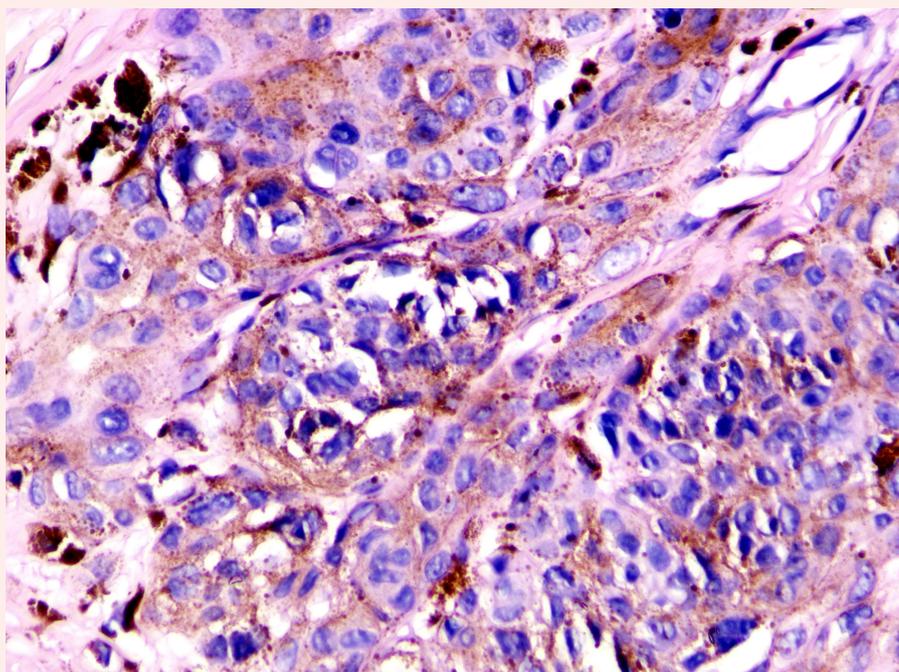


Fig. 3. Wnt-activated deep penetrating/plexiform melanocytoma. This tumor lacks maturation with descent, displays nested/plexiform melanocytes with visible nucleoli, some nuclear pleomorphism, nuclear pseudo-inclusions, and melanophages. Expresses nuclear β -catenin and LEF1.

grade areas may lack clear cell change). Perineural invasion and increased mitotic rate described. No ulceration or vascular invasion reported.

- Diffuse MART1, S100, SOX10 and MITF. Patchy HMB45. Pankeratin negative.
- Language endorsements for lesions lacking clear diagnostic criteria
 - Superficial atypical melanocytic proliferation of uncertain significance (SAMPUS) and intraepidermal atypical melanocytic proliferation of uncertain significance (IAMPUS) for lesions falling short of radial growth phase or in-situ melanoma, respectively. These designations imply virtually no risk of distant spread.
 - Melanocytic tumor of uncertain malignant potential (MELTUMP) for neoplasms where vertical growth phase of melanoma is the main alternative and thus the uncertainty lies in potential metastatic risk.

ADNEXAL TUMORS

- Updates in molecular pathology of adnexal tumors
 - Most poromas and some porocarcinomas harbor gene fusions *YAP1::MAML2* or *YAP1::NUTM1* [5]. Immunohistochemistry with NUT identifies those with *NUTM1* rearrangements
 - Some hidradenomas exhibit *CRTC1::MAML2* fusion gene.
 - *ALPK1* mutations activating NF-κB pathway in some spiradenomas and spiradenocarcinomas (mutually exclusive from *CYLD* mutations).
 - *ETV6::NTRK3* translocation and *NFIX::PKNI* fusion in cutaneous secretory carcinoma.
- Cutaneous NUT carcinoma newly introduced (provisional)
 - Rarely described *BRD3::NUTM1* or *NSD3::NUTM1* rearranged tumors [6].
 - Dermal, infiltrating neoplasm arranged in islands, cords and/or nests.
 - Glandular and squamoid differentiation with abrupt keratinization.

- Vesicular nuclei, prominent nucleoli.
- Positive NUT immunohistochemistry; CEA/EMA highlight ductules.
- Metastatic potential
- Cribriform carcinoma is renamed as cribriform tumor; definite malignant potential is unclear.

TUMORS OF THE NAIL UNIT

- Newly introduced section encompassing:
 - Onychomatricoma (Fig. 4)
 - Onychopapilloma
 - Ungual fibrokeratoma
 - Onychocytic matricoma
 - Subungual keratoacanthoma

TUMORS OF HEMATOPOIETIC AND LYMPHOID ORIGIN

- Dendritic cell neoplasms
 - Introduction of mature plasmacytoid dendritic cell proliferation (MPDCP) associated with myeloid neoplasm.
 - MPDCP is a proliferation of plasmacytoid dendritic cells with low-grade cytology occurring in patients with known myeloid neoplasms (most commonly chronic myelomonocytic leukemia and acute myeloid leukemia).
- Histiocytic neoplasms
 - ALK-positive histiocytosis: A histiocytic neoplasm that may histologically resemble juvenile xanthogranuloma and is characterized by *ALK* gene rearrangement and positive *ALK* immunohistochemistry.
- Indeterminate dendritic cell tumor replaces indeterminate dendritic cell “histiocytosis”. The definitive cell of origin remains unclear.
- *BRAF V600E* mutations in Langerhans cell histiocytosis increase risk of relapse, severe clinical manifestations and treatment failure [7]. Patients may benefit from targeted *BRAF* inhibitor therapy.
- T-cell and NK-cell lymphoproliferative disorders and neoplasms
 - Primary cutaneous T-cell lymphomas (PCTCL) are all listed as individual entities including those previously labeled “rare subtypes”, i.e., subcutaneous panniculitis-like T-cell lymphoma, extranodal NK/T-cell lymphoma, primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma, primary cutaneous γ/δ T-cell lymphoma, primary cutaneous CD4-positive small or medium T-cell lymphoproliferative disorder, and primary cutaneous acral CD8-positive lymphoproliferative disorder.
 - Cutaneous CD8-positive acral T-cell lymphoma reclassified as a lymphoproliferative disorder.

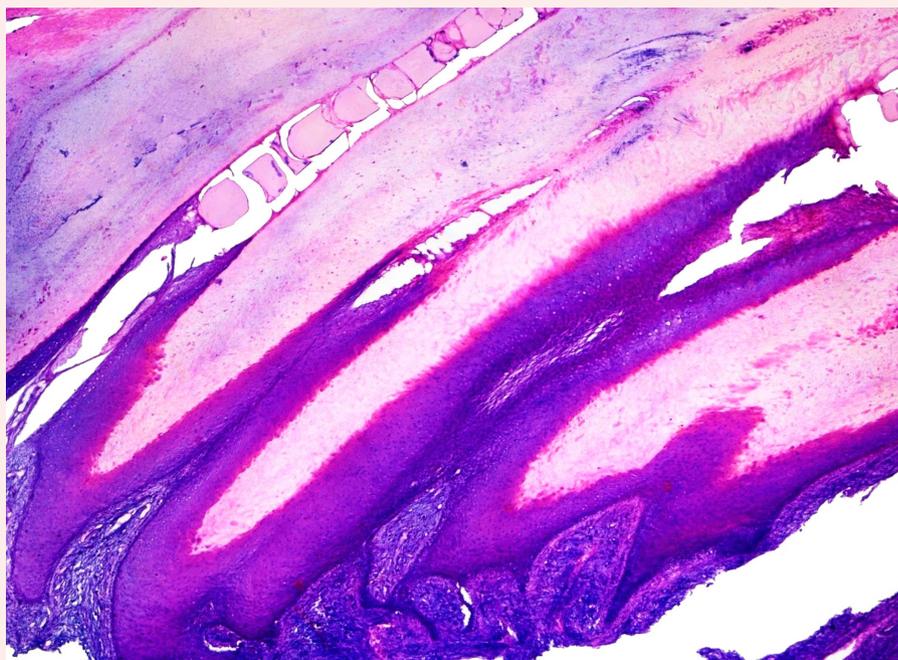


Fig. 4. Onychomatricoma. Note typical papillomatous projections of matrical-type epithelium with deep V-shaped invaginations. Subjacent spindle cell proliferation expresses CD34.

roliferative disorder. This entity is indolent with excellent long-term outcomes.

- "Primary cutaneous peripheral T-cell lymphoma, not otherwise specified (NOS)" should be used for cases that cannot be classified as a known PCTCL subtype.
- Newly introduced section on inborn error of immunity-associated lymphoproliferative disorders, which are CD8+ T-cell rich dermal infiltrates +/- granulomatous inflammation associated with inborn immunodeficiencies. May present as nodules, papules or ulceronecrotic lesions. EBV in-situ hybridization must be negative.

SOFT TISSUE TUMORS

- Introduction of four new entities:

- *CRTC1::TRIM11* cutaneous tumor: dermal-based neoplasm characterized by well-circumscribed proliferation of spindled/epithelioid cells with pale cytoplasm. Arranged in nests/fascicles and may or may not have a vaguely palisaded appearance. Diffuse SOX10 expression; variable positivity for S100 and other melanocytic markers. *CRTC1::TRIM11* fusion. Most commonly seen on the extremities. Generally indolent but may recur locally or metastasize [8,9].
- Superficial CD34-positive fibroblastic tumor: indolent neoplasm occurring in the skin and subcutis with a predilection for the extremities. Characterized by a mixture of spindled and epithelioid cells with moderate pleomorphism, prominent nucleoli, nuclear pseudoinclusions and eosinophilic cytoplasm (granular/glassy). Admixed mixed inflammatory cells are characteristic. Diffuse CD34, PRDM10, and CADM3 expression. AE1/AE3 positivity >50%. PRDM10 rearrangement in >50% of cases [10]. Excision is curative in most cases.
- *EWSR1::SMAD3* rearranged fibroblastic tumor: well-demarcated superficial tumor exhibiting intersecting

fascicles of bland fibroblastic spindle cells peripherally and relatively acellular, hyalinized collagen centrally (may lack clear zones). Diffuse nuclear ERG positivity; negative for SMA, EMA, SOX10, CD34, S100.

EWSR1::SMAD3 fusion present.

Benign tumor; may have local recurrence.

- *NTRK*-rearranged spindle cell neoplasm: group of spindle cell lesions with frequent *NTRK* rearrangements, most commonly seen in children. Involves the dermis and subcutis with a spectrum of appearances, including bland spindle cells in fibrous septa entrapping mature fat to highly cellular lesions with sheets/fascicles of spindle cells. Foci of high-grade atypia is possible. Variable CD34, SMA, S100 positivity. Diffuse pan-TRK positivity if *NTRK* fusion present.
- Atypical intradermal smooth muscle neoplasm remains the preferred terminology for smooth muscle tumors with cytologic atypia limited to the dermis. Lesions have limited metastatic potential and an excellent prognosis once completely excised.
- Epithelioid fibrous histiocytoma is classified as being of uncertain differentiation rather than a dermatofibroma subtype.

GENETIC TUMOR SYNDROMES ASSOCIATED WITH SKIN MALIGNANCIES

Newly introduced section detailing tumor syndromes with cutaneous neoplasms including:

- Familial melanoma
- *BAP1* tumor predisposition syndrome
- Xeroderma pigmentosum
- Nevoid basal cell carcinoma syndrome (Gorlin syndrome)
- Carney complex
- Muir-Torre syndrome
- Brooke-Spiegler and related syndromes

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