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*Histologic and cytologic
features of angiomatoid fibrous
histiocytoma*



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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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Front cover image: Histologic and cytologic features of angiomatoid fibrous histiocytoma, including hemorrhagic pseudocystic spaces, peripheral lymphoid aggregates, and histiocytoid tumor cells. Images are from Perez and Aung, pp. 372 and 374.

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Angiomatoid fibrous histiocytoma: a review

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Angiomatoid fibrous histiocytoma is a rare mesenchymal neoplasm of uncertain cell lineage with indeterminate behavior, hallmarked by *EWSR1* translocations. This tumor typically arises in the subcutaneous or deep soft tissues and is composed of bland to mildly atypical histiocytoid cells with frequent intralesional hemorrhagic pseudocystic spaces. It affects both children and adults, without a significant sex predilection. Histologically, the tumor may be mistaken for a lymph node given the apparent predilection for node-bearing sites as well as the brisk lymphoid cuff featuring germinal centers. Surgical excision is often curative, with local recurrence occurring occasionally and metastasis only very rarely. A possible relationship to molecularly related entities arising primarily within the thoracic cavity and intracranial compartment has been proposed, although this association remains incompletely understood.

Keywords: Histiocytoma, angiomatoid fibrous; *EWSR1*; Diagnoses, differential; *FET-CREB* family fusions

ETIOLOGY AND PATHOGENESIS

Angiomatoid fibrous histiocytoma (AFH), first described as “Angiomatoid malignant fibrous histiocytoma,” is an ultra-rare tumor of uncertain lineage [1] and is categorized by the World Health Organization as an intermediate tumor of uncertain differentiation [2]. At the time of its initial description by Enzinger in 1979 [1], it was considered a variant of what was then known as “malignant fibrous histiocytoma” (now undifferentiated pleomorphic sarcoma). However, the predominantly younger age distribution, significantly favorable prognosis, and subsequently identified recurrent molecular alteration(s) led to the recognition of AFH as a novel/distinct clinicopathologic entity.

Although AFH is now well established as a distinct tumor type, its exact histogenesis remains uncertain. The lesional cells show some morphologic resemblance to histiocytes but they lack other supporting features (including immunophenotype) indicative of true histiocytic differentiation. Other proposed

lines of differentiation have included fibroblastic reticular cell or myoid differentiation; however, no definitive conclusions have been established [3,4]. To date, there is no known precursor lesion or associated predisposing condition linked to the development of AFH.

CLINICAL FEATURES AND RADIOLOGY

The clinical and radiologic features of AFH are largely nonspecific, with most patients presenting with a slow-growing, palpable, and typically painless soft tissue mass. Clinically, these lesions may raise concern for lymphadenopathy, particularly when arising in node-bearing regions. Radiologic identification of blood-filled spaces with or without fluid-fluid levels may suggest AFH; however, these findings should be interpreted cautiously, as mimickers such as hematoma and vascular proliferations are significantly more common [5].

AFH occurs most frequently in children and young adults,

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particularly in patients younger than 30 years of age; however, cases have been reported across a wide age range, including rare congenital presentations [1,6,7]. No significant sex predilection has been identified in several large series [1,4,6]. AFH most commonly presents as a dermal, subcutaneous, or deep soft tissue mass, with the upper and lower extremities representing frequent sites of involvement. A recent study also described a cohort arising specifically in acral locations. Rarely, AFH has been reported as a primary intraosseous neoplasm [8]. Importantly, the anatomic distribution of AFH may overlap with common lymph node-bearing regions, potentially leading to clinical concern for metastatic disease from an occult primary site [9].

An uncommon but well-documented manifestation of AFH is the development of paraneoplastic inflammatory symptoms related to excess interleukin-6 (IL-6) production by the tumor. In tumors harboring CREB1 (cAMP response element-binding protein 1)-related fusion transcripts, constitutive activation

of transcriptional pathways is thought to promote IL-6 overexpression through multiple transcription factor binding sites within the IL-6 gene promoter region [10-13]. Associated systemic manifestations may include fever, weight loss, anemia, malaise, and other “B symptoms,” resembling those encountered in other neoplastic and inflammatory disorders with paraneoplastic syndromes [10].

PATHOLOGIC FINDINGS

Grossly, AFH typically presents as a well-circumscribed, firm soft tissue mass, with some cases demonstrating hemorrhagic cut surfaces. Histologically, the most characteristic and consistently observed features include sheets or fascicles of spindled-to-histiocytoid cells, central hemorrhagic or pseudocystic spaces, and a lymphoid cuff composed predominantly of chronic inflammatory cells, findings that are present in the majority, but not all, of cases (Fig. 1) [3,6,14,15]. Tumor cells

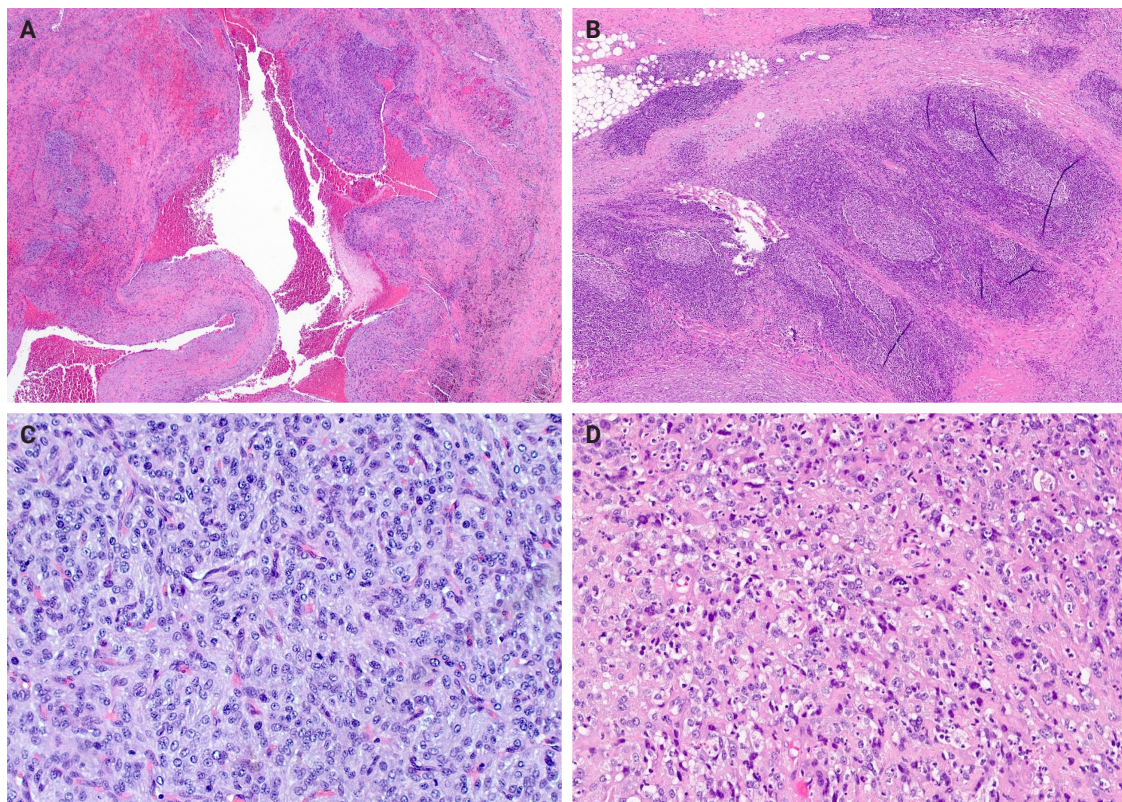


Fig. 1. Histologic features of angiomatoid fibrous histiocytoma. (A) Low power image demonstrating the cystic appearing spaces with prominent blood. (B) A mid-power image demonstrates conspicuous lymphoid aggregates with germinal center formation, seen commonly at the periphery of the lesion. (C) A high-power image shows the typical cytologic features, with sheets of histiocytoid cells without significant atypia. (D) Some foci show increased cytologic atypia, which is of uncertain clinical significance and can be focal in nature.

are generally plump and are often arranged in syncytial sheets. At the periphery of the lesion, there is frequently a dense rim of lymphocyte-rich chronic inflammatory infiltrate with well-formed germinal centers. This feature may lead to diagnostic confusion with a lymph node or lymphoid neoplasm [1,4,6].

Mitotic activity is generally low or absent; however, a small subset of cases demonstrates increased mitotic activity (>5 mitoses per 10 high-power fields). Some studies have suggested a possible association between elevated mitotic activity and an increased risk of local recurrence or metastasis, although a definitive prognostic correlation has not been established [6,14,15]. Additionally, focal or scattered pleomorphic tumor cells may occasionally be identified, though the biologic significance of this finding remains uncertain [14,16]. Similar to other neoplasms of intermediate biologic potential, local recurrence appears to be more strongly associated with incomplete excision than with cytologic atypia [6].

Immunohistochemically, AFH demonstrates a relatively characteristic but nonspecific immunophenotype (Table 1).

Most tumors express variable combinations of desmin, epithelial membrane antigen (EMA), CD68, CD99, and calponin, with each marker reported in approximately half of cases (Fig. 2). Despite the relatively frequent expression of desmin and calponin, there is no convincing evidence supporting true skeletal muscle differentiation, as MyoD1 and myogenin are consistent-

Table 1. Frequency of immunohistochemical marker expression in angiomatoid fibrous histiocytoma

Marker	Frequency (%)
CD99	50–90
Desmin	50 to >90
EMA	>70
CD68	~50
Calponin	73 (focal) and 12 (diffuse)
ALK	>80
MUC4	~20

Data summarized from PathologyOutlines.com (<https://www.pathologyoutlines.com/topic/skintumornonmelanocyticafh.html>) [17].

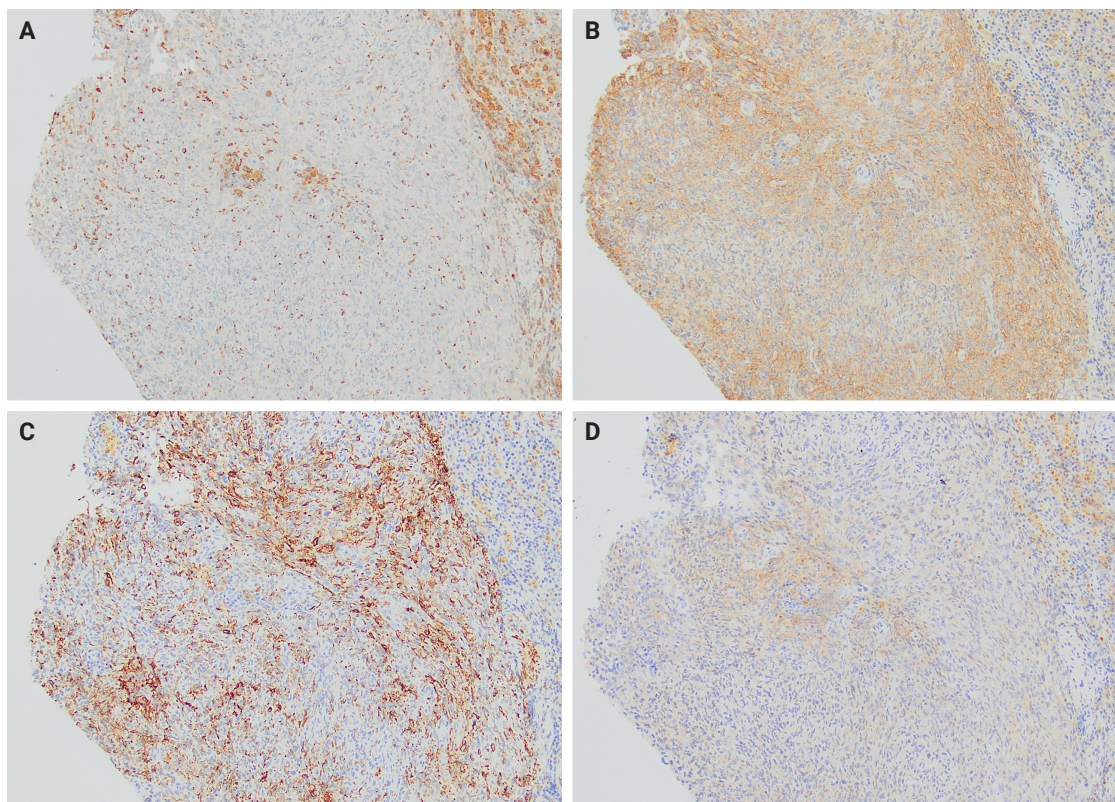


Fig. 2. Immunohistochemical pattern of angiomatoid fibrous histiocytoma. (A) CD68 shows scattered expression in lesional cells, as well as histiocytes within the wall (right). (B) CD99 shows patchy expression which is predominantly cytoplasmic in nature. Desmin (C) expression is patchy but strong, while epithelial membrane antigen (D) can be weaker and more focal.

ly negative [4].

Additional immunohistochemical markers have been investigated in recent years. Anaplastic lymphoma kinase (ALK) expression, particularly with the D5F3 clone, has been reported in a subset of AFH cases despite the absence of corresponding *ALK* gene fusions/rearrangements or copy number alterations [18-20]. Furthermore, variable MUC4 expression has been identified in a minority of tumors [21]. Awareness of these staining patterns is important to avoid potential diagnostic confusion with inflammatory myofibroblastic tumor in ALK-positive cases or with low-grade fibromyxoid sarcoma/sclerosing epithelioid fibrosarcoma in MUC4-positive lesions.

MOLECULAR AND CYTOGENETICS

The FET family of RNA-binding proteins, comprising *EWSR1* (Ewing sarcoma breakpoint region 1), *FUS* (fused in sarcoma), and *TAF15* (TATA-box binding protein-associated factor 15), plays a central role in the pathogenesis of numerous fusion-driven mesenchymal neoplasms. Members of this family frequently function as interchangeable fusion partners in a wide variety of sarcomas and other soft tissue tumors. Early molecular studies of AFH identified *EWSR1::ATF1* and *FUS::ATF1* fusion events. However, subsequent investigations have demonstrated that the most common molecular abnormality in AFH is the *EWSR1::CREB1* fusion, most frequently involving exon 7 of *EWSR1* fused to exon 7 of *CREB1* [14,22,23]. Less commonly, alternative fusion partners have been described, including

rare variant fusions such as *EWSR1::PBX3*, which nevertheless appear to retain morphologic features characteristic of conventional AFH [14].

The cyclic AMP response element-binding protein (CREB) family of transcription factors is involved in the regulation of cellular proliferation, differentiation, and survival. Aberrant activation of CREB-family transcription factors, whether through fusion events, constitutive activation, or other molecular alterations, has been implicated in the pathogenesis of a broad spectrum of neoplasms, including hematologic malignancies and sarcomas [11]. In AFH, fusion-driven activation of CREB-family members is believed to contribute to tumorigenesis through dysregulated transcriptional signaling pathways.

Given the presence of overlapping molecular alterations, particularly involving *EWSR1* and *CREB*-family fusion partners, AFH shares molecular similarities with several other neoplasms. These relationships have prompted comparisons with a variety of tumors arising in soft tissue, visceral, and intracranial locations, which are discussed in the Differential Diagnosis section below.

DIFFERENTIAL DIAGNOSIS (AND POSSIBLY RELATED ENTITIES)

Morphologically, the differential diagnosis for AFH includes both reactive and neoplastic entities. Reactive conditions such as non-necrotizing granulomatous lymphadenitis may enter the differential diagnosis, particularly when AFH arises in nodal

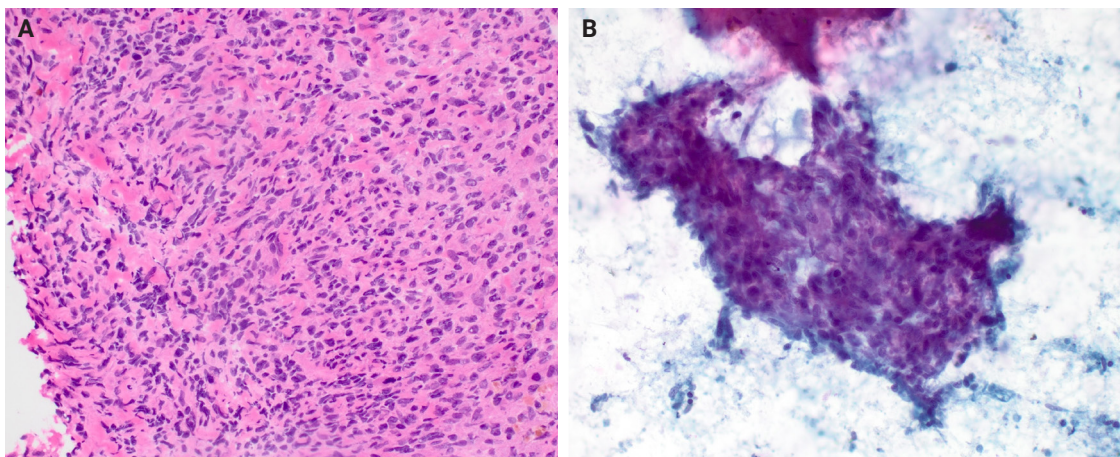


Fig. 3. Additional tissue preparations showing angiomatoid fibrous histiocytoma. A high-power view on an intraoperative frozen section slide (A) demonstrates that the cells can appear confluent, resembling a granuloma which is similar to the appearance by cytologic preparations (Papanicolaou stain, B) which also could be construed as histiocytic cells.

or peri-nodal locations, which can confound examination by intraoperative or cytologic means (Fig. 3). However, granulomatous lesions typically contain necrotic or fibrinoid central material rather than the hemorrhagic pseudocystic spaces characteristic of AFH. Among neoplastic mimickers, follicular dendritic cell sarcoma (FDSC) may show overlapping features, including a lymphoid-rich background. Nevertheless, FDSC more commonly demonstrates uniform cytologic atypia, fascicular architecture, and inflammation distributed throughout the tumor rather than confined to the periphery, as seen in AFH. Immunohistochemically, AFH may be distinguished by expression of desmin and/or EMA and lack of follicular dendritic cell markers such as CD21, CD23, and CD35.

Vascular neoplasms, particularly epithelioid hemangioma (formerly termed angiolymphoid hyperplasia with eosinophilia), may also be considered in cases with prominent hemorrhage. In contrast to AFH, epithelioid hemangioma demonstrates well-formed vascular channels and generally lacks the solid cellularity and syncytial growth pattern characteristic of AFH. Endothelial markers such as ERG and CD31 are helpful in excluding vascular neoplasia.

In atypical cases, particularly those with increased cytologic atypia or EMA expression, AFH may raise concern for metastatic carcinoma of unknown primary origin. Recognition of the generally bland cytomorphology of AFH, combined with appropriate clinicopathologic correlation and exclusion of a known primary epithelial malignancy, is essential in avoiding this diagnostic pitfall. Perhaps the most important histologic mimicker of AFH is aneurysmal fibrous histiocytoma (also termed aneurysmal dermatofibroma), a variant of cellular fibrous histiocytoma. Like AFH, these lesions may exhibit extensive hemorrhagic change and hemosiderin deposition. However, aneurysmal fibrous histiocytoma typically demonstrates slender spindle cells arranged in a storiform pattern, rather than the sheet-like or syncytial architecture seen in AFH. In addition, the dense peripheral lymphoid cuff characteristic of AFH is generally absent. Clinically, aneurysmal fibrous histiocytoma behaves in an overwhelmingly indolent manner, although rare metastatic cases have been reported [24,25].

The molecular profile of AFH, particularly its recurrent *FET-CREB* family gene fusions, has raised the possibility of biologic relationships with several neoplasms arising in other anatomic sites. One such entity is primary pulmonary myxoid sarcoma (PPMS), a rare pulmonary neoplasm characterized by cords, reticular growth, and myxoid stroma [26]. Some cases of PPMS

exhibit morphologic overlap with AFH, including syncytial architecture and peripheral lymphoid cuffs. However, PPMS generally demonstrates more rounded or slender spindle cells within a distinctly myxoid background. Immunohistochemically, EMA expression may be seen in occasional cases, although desmin expression is typically absent. The presence of *EWSR1::CREB1* fusions in most PPMS cases has led to speculation that these lesions may represent part of a related biologic spectrum [27].

Within the central nervous system, there is a recently described “Intracranial mesenchymal tumor with *FET-CREB* fusion,” which shares substantial morphologic and molecular overlap with AFH. These tumors may arise intracranially or within the spinal canal and can closely resemble AFH histologically. Interestingly, a female predominance has been reported, with some studies suggesting ratios approaching 4:1. Larger series have demonstrated potential correlations between specific fusion types and morphologic patterns, and epigenetic profiling has suggested possible subclassification schemes. A minority of cases have additionally shown rearrangements involving members of the *SMARCB1/SMARCA* family, alterations not typically identified in conventional AFH [28,29]. Given the marked overlap in morphology and cytogenetic features, these tumors are widely considered to be closely related to AFH.

Other neoplasms harboring similar molecular alterations are generally more readily distinguished from AFH based on morphology and immunophenotype. Clear cell sarcoma, most commonly associated with the *EWSR1::ATF1* fusion, demonstrates nests and fascicles of uniform spindle-to-epithelioid cells with clear cytoplasm, prominent nucleoli, and sclerotic stroma. These tumors also show diffuse melanocytic differentiation, with strong expression of S100, SOX10, and HMB45 [30]. In addition, emerging reports have described intra-abdominal malignant epithelioid neoplasms with *FUS::CREM* rearrangements, which are characteristically strongly and diffusely positive for cytokeratins and lack significant morphologic overlap with AFH [31].

Other uncommon considerations include inflammatory myofibroblastic tumor, myoepithelial neoplasms with *EWSR1* rearrangements, synovial sarcoma with lymphoid-rich stroma, and, rarely, *CIC*-rearranged sarcoma, in the appropriate clinicopathologic context.

TREATMENT AND PROGNOSIS

Angiomatoid fibrous histiocytoma (AFH) is classified as an intermediate (rarely metastasizing) neoplasm with indeterminate biologic potential, reflecting its tendency for local recurrence but overall low metastatic risk. Accordingly, complete surgical excision remains the mainstay of treatment [32]. Local recurrence represents the most common adverse clinical outcome, although it occurs relatively infrequently, reported in fewer than 10% of cases and as low as approximately 1% in some series. Metastatic disease is distinctly uncommon but has been documented in rare instances, involving both regional lymph nodes and the lungs [4,6,24,33].

As discussed previously, a subset of AFH cases demonstrates excess IL-6 production, resulting in paraneoplastic inflammatory symptoms. In this setting, IL-6–targeted therapies have been utilized for symptom control, with variable reported effectiveness regarding both systemic symptom improvement and tumor response [12,13,34].

In unresectable cases or in patients for whom surgery is not feasible, systemic chemotherapy has been employed in small case series and reports, with outcomes ranging from partial response to complete radiologic remission at primary and/or metastatic sites [35]. Additionally, although ALK expression has been identified immunohistochemically in a subset of AFH cases, corresponding *ALK* gene rearrangements are generally absent. Nevertheless, isolated reports have suggested that ALK expression may carry therapeutic relevance, and ALK-targeted agents, similar to those used in ALK-positive non-small cell lung carcinoma, have been administered in metastatic AFH [36].

More recently, programmed death-ligand 1 expression has been investigated in AFH, with at least focal positivity identified in a majority of tested cases (approximately 60% of reported cases). Although the clinical significance of this finding remains uncertain, it raises the possibility that immune checkpoint inhibitor therapy could represent a future therapeutic option, particularly in advanced, unresectable, or metastatic disease settings where conventional surgical management is not feasible.

CONCLUSION

Angiomatoid fibrous histiocytoma (AFH) is a rare mesenchymal neoplasm of uncertain differentiation that most commonly

arises within the subcutaneous soft tissues of children and young adults, although it may occur across a broad age range and at diverse anatomic sites. Despite its generally indolent clinical course, AFH is classified as an intermediate (rarely metastasizing) tumor because of its recognized potential for local recurrence—particularly following incomplete excision—and its low but well-documented metastatic risk.

Diagnosis of AFH relies on the integration of morphologic, immunohistochemical, and molecular findings. The differential diagnosis encompasses a variety of reactive and neoplastic entities, including tumors with overlapping histologic features as well as lesions sharing similar molecular alterations involving *FET-CREB* family fusions. In most cases, careful histopathologic evaluation combined with appropriate immunohistochemical, and molecular studies allows accurate distinction from these mimickers.

Continued investigation into the molecular landscape and biologic relationships of AFH may further clarify its histogenesis and potential connections with other *FET-CREB* fusion-associated neoplasms, while also opening avenues for targeted therapeutic strategies in advanced or unresectable disease.

Ethics Statement

Not applicable.

Availability of Data and Material

Data sharing not applicable to this article as no datasets were generated or analyzed during the study.

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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Ganglioglioma and gangliocytoma: a review for pathologists

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Ganglioglioma and gangliocytoma are rare, predominantly low-grade neuroepithelial tumors that commonly present with epilepsy in children and young adults. Advances in molecular profiling have improved understanding of their pathogenesis, highlighting key roles for the mitogen-activated protein kinase/ERK signaling pathway. Diagnosis relies on a combination of clinical, radiologic, and histopathologic features, with complete surgical resection offering the best clinical outcomes. This review summarizes current knowledge on their epidemiology, etiology, clinical presentation, imaging characteristics, pathology, treatment strategies, and prognosis.

Keywords: Ganglioglioma; Seizures; Epilepsy; Neuropathology; Proto-oncogene proteins B-raf

INTRODUCTION

Ganglioglioma and gangliocytoma are rare, low-grade central nervous system (CNS) tumors that primarily affect children and young adults. Patients with these lesions often present with seizures, particularly when located in the temporal lobe. Gangliogliomas are more frequently encountered and are recognized as a leading cause of epilepsy-associated brain tumors in both pediatric and adult populations [1-3]. Gangliocytomas, while less common, share many clinical and histopathological features with gangliogliomas. Despite their relative rarity, these tumors are clinically significant due to their epileptogenic potential and generally favorable outcomes following appropriate management. This review provides a structured overview of gangliogliomas and gangliocytomas, addressing key aspects of their epidemiology, pathogenesis, clinical presentation, neuroimaging characteristics, histopathologic features, molecular properties, therapeutic approaches, and prognostic implications.

EPIDEMIOLOGY

Gangliogliomas have an estimated global incidence of 0.186 cases per 100,000 population [4]. They exhibit a slight male predominance [4-6] and account for approximately 0.4% of all CNS tumors and 1%–7% of pediatric CNS neoplasms [7,8]. These tumors predominantly affect children and young adults, with the majority of cases diagnosed before the age of 20 and a median age of 12 years, although they can occasionally present in older individuals [1]. In epilepsy surgical series, gangliogliomas represent the most frequent histopathological diagnosis among brain tumors associated with intractable epilepsy in adult and pediatric patients, accounting for approximately 40% of such cases [1-3]. Gangliocytomas are much less common, with an incidence of 0.016 cases per 100,000 population [4]. In the context of epilepsy-related surgical resections, gangliocytomas account for approximately 1%–3% of cases [1,9].

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ETIOLOGY AND PATHOPHYSIOLOGY

Gangliogliomas result from genetic alterations that activate the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) signaling cascade, including proteins such as MEK, ERK, RSK1, RHEB, and MSK1, which collectively promote aberrant cell proliferation and oncogenesis [10,11]. Although most gangliogliomas occur sporadically, a minority are associated with neurofibromatosis type 1 (NF1) [12]. In addition, gangliogliomas may coexist with adjacent focal cortical dysplasia (FCD), particularly FCD type III [13]. While molecular data regarding gangliocytomas remain limited, their overlapping histopathologic and clinical features with gangliogliomas suggest a shared pathophysiologic basis. Dysplastic cerebellar gangliocytoma (Lhermitte-Duclos disease) associated with Cowden syndrome is considered a distinct pathologic entity and will not be included in the discussion here (for comprehensive review, see Alanazi et al. [14]). To date, no definitive environmental risk factors have been identified for either tumor type.

CLINICAL FEATURES

The clinical presentation of gangliogliomas and gangliocytomas is closely linked to their anatomical location, with lesions in the temporal lobe of the cerebrum frequently associated with seizures and epilepsy. Studies have consistently reported epileptic crises as the most common initial manifestation [5,15], with an average symptom duration of 7.4 years prior to diagnosis [16]. Additional symptoms may include headache, hydrocephalus, and developmental disorders [17].

RADIOLOGY

Gangliogliomas are typically characterized on brain imaging as circumscribed, cystic masses with an avidly enhancing mural nodule. On magnetic resonance imaging, the cystic component is classically T1 hypointense and T2 hyperintense [18,19]. These tumors often exhibit minimal to no surrounding edema and lack significant mass effect, contributing to their well-delimited appearance. Calcifications are common, present in approximately 40%–50% of cases, and enhancement patterns can vary, especially in less typical presentations where the lesion may appear as a solid mass. The most frequent location is the temporal lobe, followed by the frontal, occipital, and parietal lobes; less commonly, gangliogliomas may arise in the cere-

bellum, brainstem, or spinal cord [5,18]. Additionally, areas of cortical dysplasia are often observed adjacent to the tumor [18].

PATHOLOGIC FINDINGS

Gross findings

Gangliogliomas and gangliocytomas are typically grossly well-demarcated lesions, often presenting as variably solid and cystic masses. These tumors characteristically lack areas of hemorrhage and necrosis.

Frozen section findings

Intraoperative frozen section evaluation reveals a similarly well-circumscribed mass; however, a diffuse growth pattern involving adjacent brain parenchyma may also be observed, especially at the tumor edge. On a frozen preparation, gangliogliomas exhibit a biphasic cellular composition, comprising dysplastic ganglioid cells intermixed with atypical glial elements (Fig. 1A–C), whereas gangliocytomas are predominantly composed of neuron-like cells. Additional features include variable microcalcifications (Fig. 1C), perivascular lymphocytic infiltrates, and eosinophilic granular bodies (Fig. 1B). Squash preparations of gangliogliomas demonstrate glial cells with fine, piloid-like processes and dysplastic ganglioid cells embedded within a vascular network of thin- to thick-walled vessels (Fig. 1D–F) [20].

Microscopic findings

Histologically, ganglioglioma is a biphasic tumor characterized by a variable admixture of dysmorphic ganglion-like cells and atypical glial elements, generally well-demarcated from the adjacent brain. A representative case with classic histopathologic features is presented in Fig. 2. Microscopically, the dysplastic ganglion-like cells exhibit several hallmark features, including binucleation, cytomegaly with ballooned cytoplasm, and peripheral accumulation of Nissl substance. These cells often form abnormal clusters, referred to as "kissing neurons," and lack an organized cytoarchitecture (Fig. 2C) [5,15]. The glial component comprises hyperchromatic, moderately enlarged cells that may resemble those seen in fibrillary astrocytomas, pilocytic astrocytomas, or oligodendrogliomas [5,21]. Eosinophilic granular bodies are more frequently observed than Rosenthal fibers, and additional features include dystrophic calcifications, perivascular lymphoid infiltrates, and a prominent capillary network. Less common histologic findings include prominent

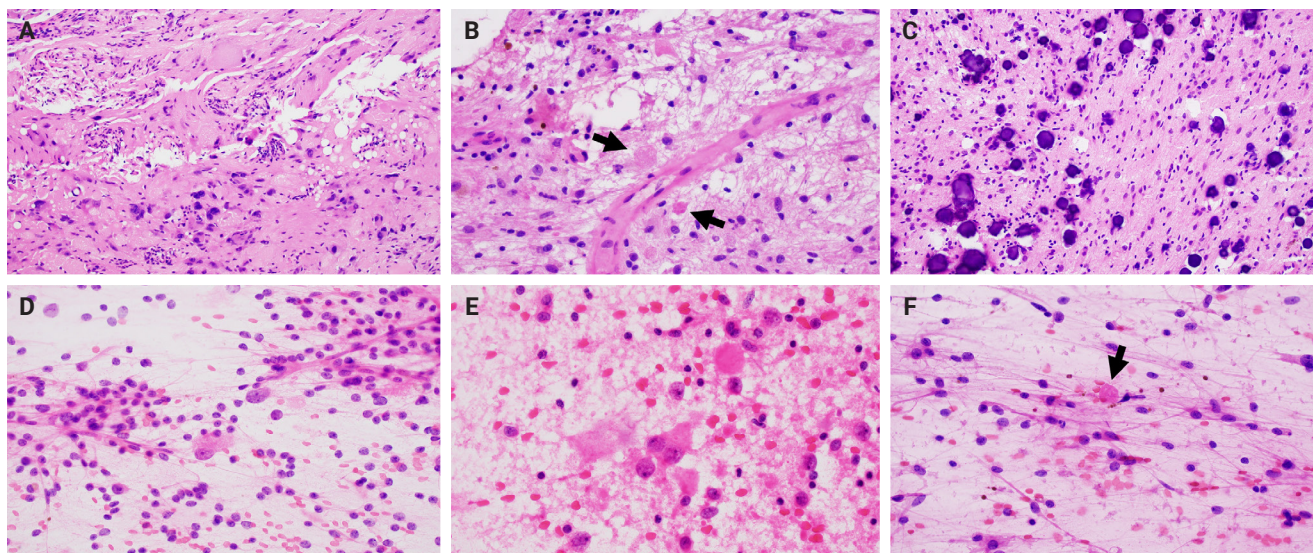


Fig. 1. Ganglioglioma/gangliocytoma features at intraoperative consultation. (A–C) Frozen sections reveal moderately hypercellular tissue composed of an admixture of atypical and abnormally aggregated glial and ganglioid elements. The background may have piloid elements, eosinophilic granular bodies (EGBs, arrows), and/or microcalcifications. (D–F) Smear preparation reveals a monomorphic population of enlarged glial cells with round to elongated nuclei and variably hyperchromatic chromatin and thin, fibrillary cellular processes. Dysplastic ganglioid cells may be encountered, including cells with abnormally dispersed chromatin and/or binucleation. Careful examination can also reveal EGBs (arrow).

piloid histology, perinuclear halos (i.e., “oligo-like” morphology), and nuclear pleomorphism (Fig. 3). Mitotic activity is typically low to absent [5]. Microscopic infiltration along the tumor edge may be present despite its generally compact growth pattern (Fig. 2B) [1]. Rarely, gangliogliomas may exhibit anaplastic features such as increased mitotic figures, microvascular proliferation, and necrosis. However, the designation “anaplastic ganglioglioma” should be applied cautiously, particularly in the absence of comprehensive molecular characterization to exclude other high-grade gliomas with similar histomorphology, including pleomorphic xanthoastrocytoma [22–24]. In contrast to gangliogliomas, gangliocytomas are composed almost exclusively of large, dysmorphic ganglion cells and lack the neoplastic glial component that characterizes the former; a representative case is presented in Fig. 4.

Immunohistochemistry

Ganglion-like cells within gangliogliomas and gangliocytomas express neural markers such as MAP2, neurofilament, and synaptophysin (Figs. 2D, E, 4E). They often lack NeuN immunoreactivity, which is expressed in adjacent non-neoplastic neurons. The glial component of these tumors demonstrates positivity for glial fibrillary acidic protein and Olig2 (Fig. 2F, G) [25].

CD34 expression, either diffuse or patchy in an arborescent pattern, is frequently observed within the tumor or adjacent cortex (Fig. 2H) [26] and may serve as a supportive diagnostic feature in diagnostically challenging cases. Proliferative activity is generally low, with Ki67 indices typically less than 5% [27]. A *BRAF* V600E mutation-specific antibody is available and often positive in dysplastic ganglion-like cells [28]. These tumors are negative for *IDH1* (isocitrate dehydrogenase type 1) R132H and display retained nuclear ATRX expression, distinguishing them from other gliomas with IDH mutations.

MOLECULAR AND CYTOGENETICS

Gangliogliomas exhibit a diverse range of molecular alterations, with the *BRAF* V600E mutation being the most common, identified in approximately 10%–60% of cases. Its prevalence is highest in cortical tumors and lowest in spinal cord lesions [21,29,30]. Additional genetic abnormalities include other *BRAF* mutations, *BRAF* gene fusions (most frequently *KIAA1549::BRAF*), *KRAS* activating mutations, and *NF1* inactivating mutations or deletions [31]. The *BRAF* V600E mutation can be detected via immunohistochemistry or molecular techniques such as gene sequencing, including droplet digital polymerase chain reaction

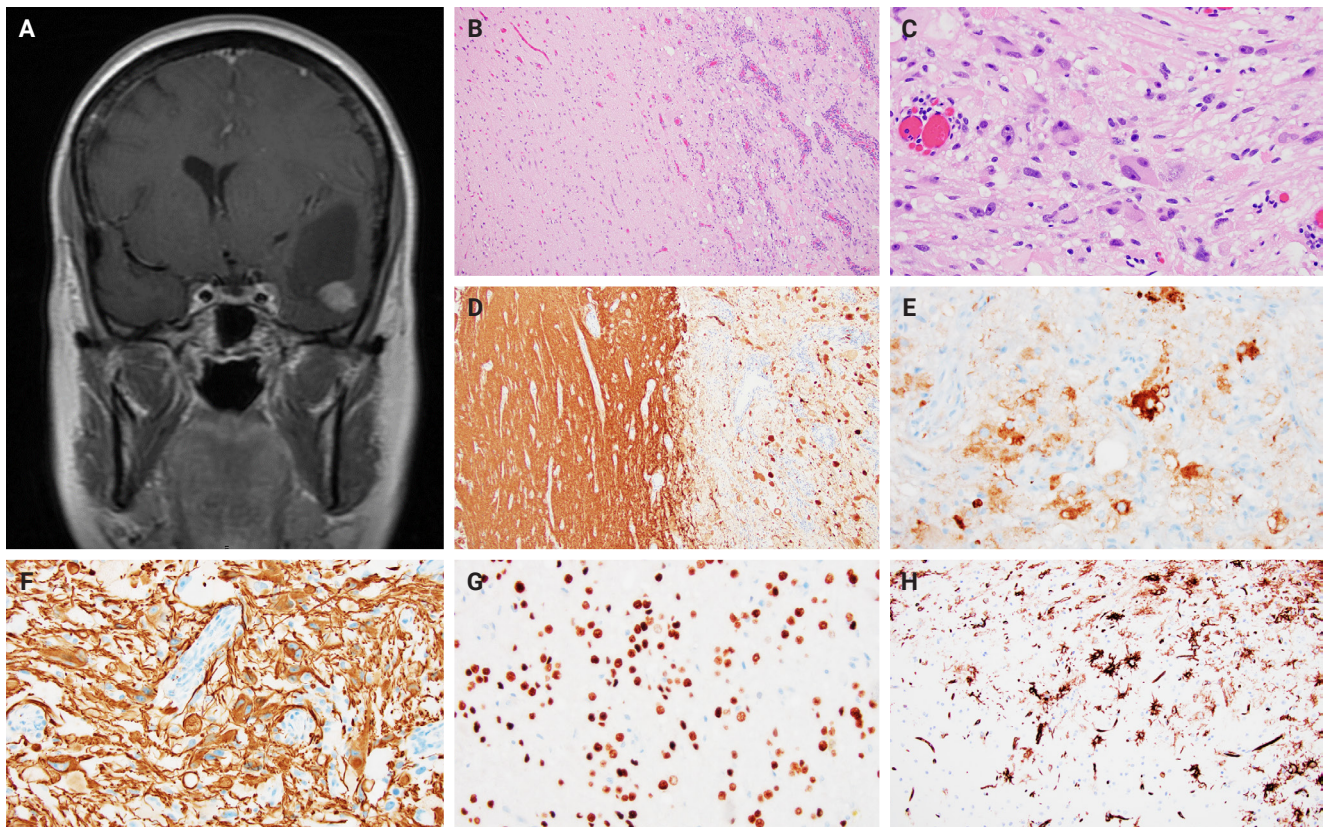


Fig. 2. Representative findings in ganglioglioma. (A) Coronal magnetic resonance imaging reveals a cystic lesion with an enhancing mural nodule located in the left temporal lobe. (B) Low magnification hematoxylin and eosin (H&E) reveals a mildly hypercellular tumor (image right), well-demarcated from the adjacent brain (image left). (C) High magnification H&E reveals a biphasic tumor composed of an admixture of mildly atypical and hyperchromatic glial cells and multinucleated ganglioid cells. Immunohistochemistry reveals synaptophysin-positive ganglioid cells (D, E) and glial cells that are positive for glial fibrillary acidic protein (F) and Olig2 (G). CD34 immunostain highlights ramified elements within and adjacent to the tumor (H).

[28]. In cases negative for *BRAF* V600E, broader molecular testing using next-generation sequencing or gene fusion panels is recommended to identify other MAPK pathway-activating alterations [31]. Furthermore, gangliogliomas demonstrate a distinct genome-wide DNA methylation profile, which can aid in diagnosis, particularly in histologically ambiguous cases [32]. However, despite the high accuracy of DNA methylation profiling, gangliogliomas may remain difficult to classify, comprising 8.7% of tumors lacking confident methylation-based classification [33]. Gangliocytomas currently lack a defined molecular signature, and no characteristic genetic profile has been published to date.

DIFFERENTIAL DIAGNOSIS

Distinguishing the various cortical-based neuroepithelial lesions

requires careful integration of clinical, radiologic, histopathologic, immunohistochemical, and molecular features. FCD typically lacks well-circumscribed tumor architecture and retains NeuN immunoreactivity in dysplastic neurons, with genetic alterations often involving the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin pathway. The presence of MAPK activating alterations (such as *BRAF* V600E) can be helpful to exclude this entity [13,34]. In contrast, infiltrating low-grade gliomas demonstrate entrapped NeuN-positive neurons within a neoplastic glial population and frequently harbor *IDH1/IDH2* mutations. In adolescents and young adults, tumors with diffuse architecture may harbor MAPK pathway alterations (i.e., “Diffuse low-grade glioma, MAPK pathway-altered”), but dysplastic ganglion cells are absent. Polymorphous low-grade neuroepithelial tumor of youth (PLNTY) is characterized by *FGFR2* (fibroblast growth factor receptor 2)/*FGFR3* (fibroblast growth

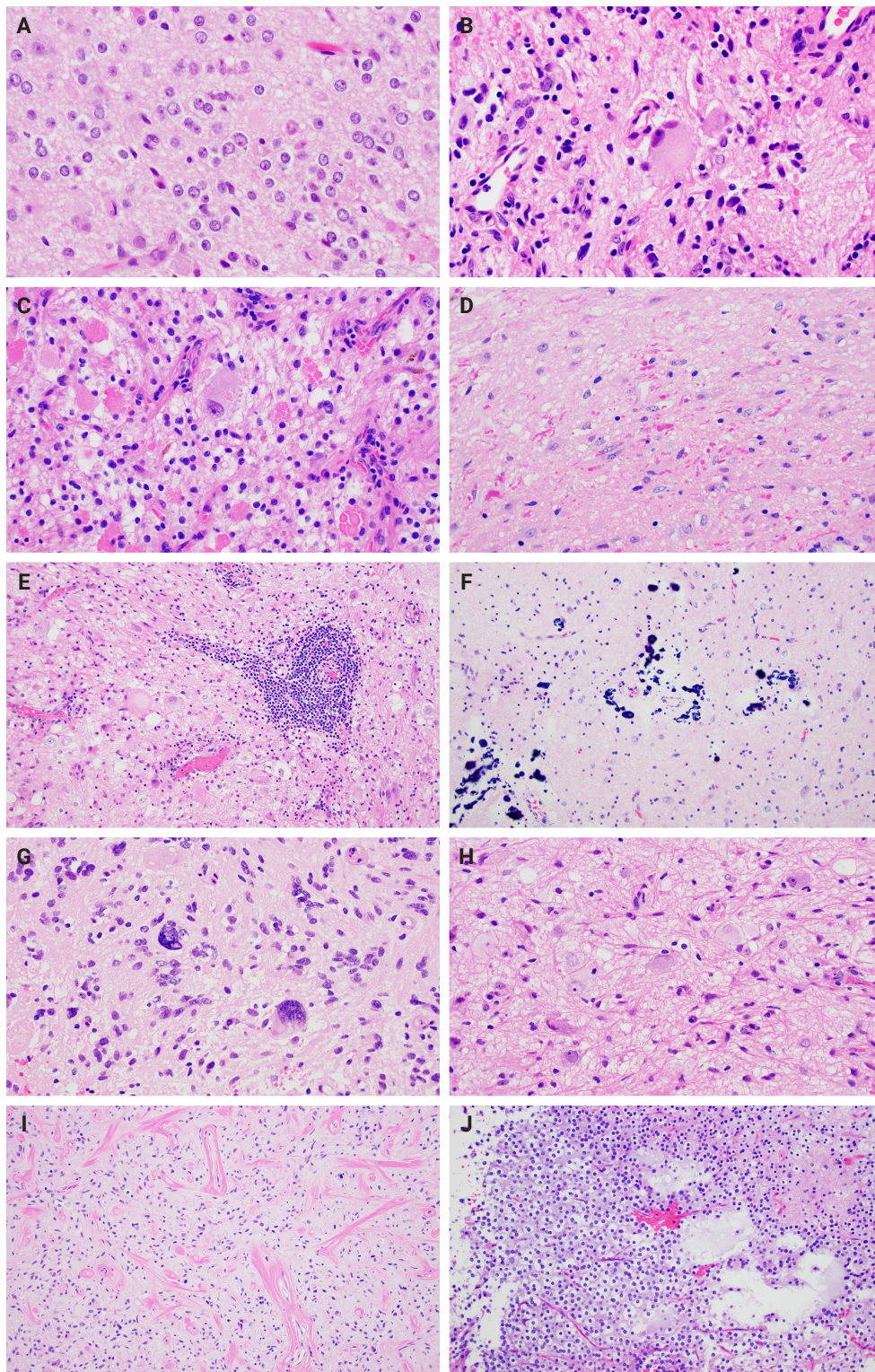


Fig. 3. Additional histopathologic findings in ganglioglioma. In some cases, the glial elements are much more prominent (A), and dysplastic ganglioid cells are only focally present (B). Eosinophilic granular bodies can be multiple and prominent (C), whereas Rosenthal fibers are not commonly encountered (D). Perivascular lymphocytic cuffing (E) and microcalcifications (F) are found in many cases. Some tumors may demonstrate nuclear pleomorphism (G), extensive piloid background (H), prominent hyalinized vasculature (I), and/or tumor cells with round nuclei and perinuclear halos ("oligo-like") (J).

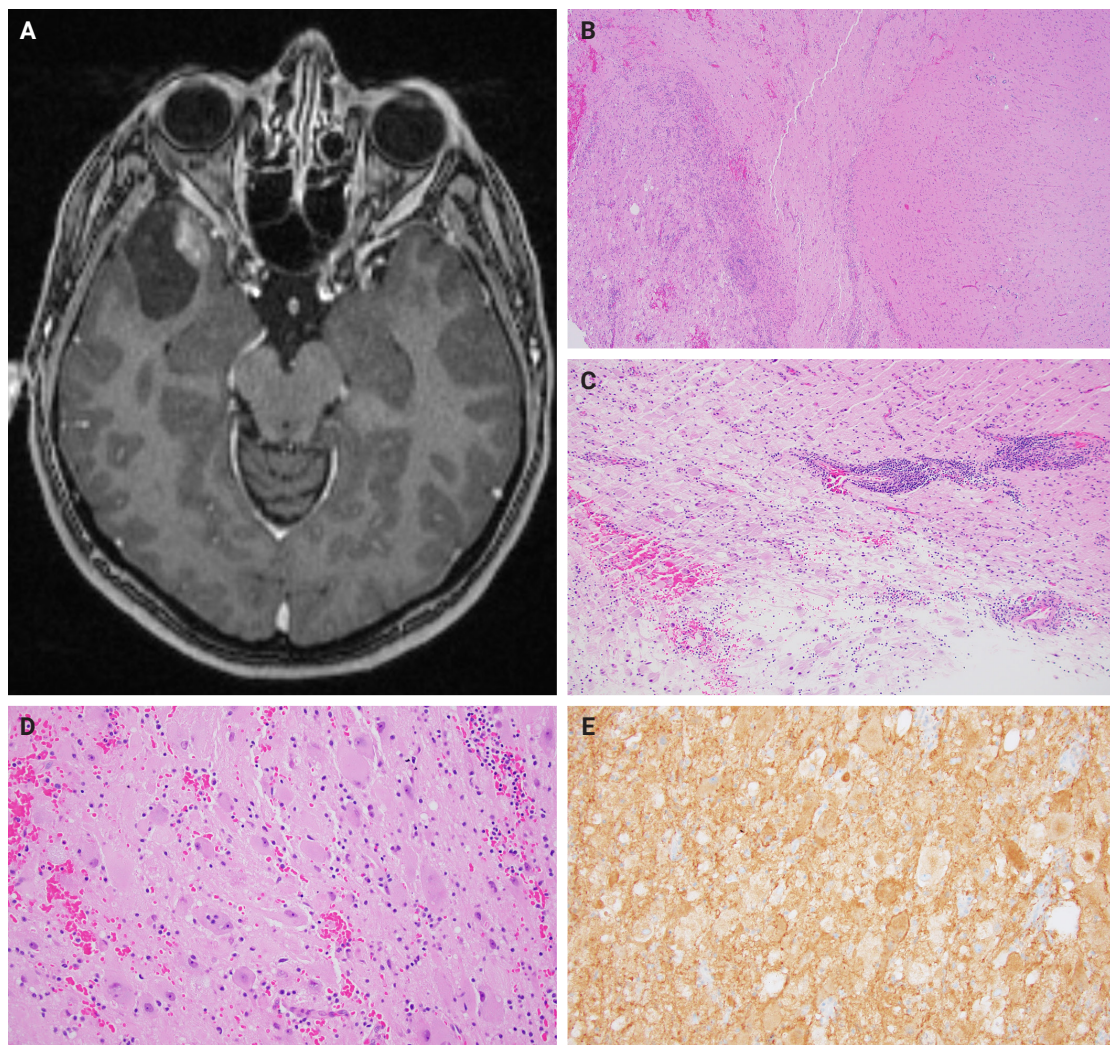


Fig. 4. Representative findings in gangliocytoma. (A) Sagittal magnetic resonance imaging reveals a solid and cystic mass within the anterior right temporal lobe with an enhancing mural nodule. (B) Low magnification hematoxylin and eosin (H&E) reveals a mildly hypercellular tumor (image left), well-demarcated from the adjacent brain (image right). (C) There are areas with prominent perivascular lymphocytic cuffs. (D) High magnification H&E reveals a tumor composed nearly entirely of dysplastic ganglioid cells with abundant eosinophilic cytoplasm and occasional bi/multinucleated forms. (E) Tumor cells are uniformly immune-positive for synaptophysin.

factor receptor 3) fusions or *BRAF* V600E mutations, diffuse CD34 positivity, and focally infiltrative oligodendroglial-like components [35], though significant overlap may exist between PLNTY and ganglioglioma. Dysembryoplastic neuroepithelial tumors show multinodular architecture with floating neurons in a mucinous matrix and frequent *FGFR1* alterations and typically lack CD34 expression [36]. Multinodular vacuolating neural tumors also exhibit multinodular growth, prominent vacuolization, and Olig2-positive neuronal cells, but lack dysmorphic neurons and CD34 staining [37]. Pleomorphic xanthoastrocytoma presents with xanthomatous cells, nuclear pleomorphism,

and a reticulin-rich stroma, often harboring both *BRAF* V600E mutation and *CDKN2A/CDKN2B* homozygous deletion [38]. Desmoplastic infantile ganglioglioma is distinguished by its large size in infantile patients and a markedly desmoplastic stroma [39]. Hypothalamic hamartomas, pathognomonic for Pallister-Hall syndrome, are congenital lesions presenting with gelastic seizures and *GLI3* mutations [40,41]. Lastly, dysplastic cerebellar gangliocytoma (Lhermitte-Duclos disease) is associated with Cowden syndrome and *PTEN* loss and lacks *MAPK* alterations typical of gangliogliomas [42].

TREATMENT AND PROGNOSIS

The management of gangliogliomas and gangliocytomas centers on surgical intervention, with complete resection representing the cornerstone of treatment and the most significant prognostic factor, particularly for gangliogliomas [7]. Gangliogliomas are low-grade tumors associated with excellent long-term outcomes, with reported 15-year overall survival rates ranging from 83% to 94%. In cases that are progressive, recurrent, or refractory, targeted therapies such as RAF or MEK inhibitors have shown potential benefit, although their clinical utility remains under investigation [43-46]. Emerging molecular data suggest that certain genetic profiles, including co-occurrence of *BRAF* V600E with *CDKN2A/CDKN2B* homozygous deletions or midline tumors harboring both *BRAF* V600E and *H3* K27M mutations, may be associated with poorer outcomes [31,47-49]. Another report suggests tumors with *PTPN11/KRAS/NF1* and other MAPK pathway alterations may show atypical histologic features and adverse clinical outcomes [50]. Similarly, gangliocytomas are benign lesions with excellent long-term survival, and to date, specific prognostic markers have not been identified.

CONCLUSION

Gangliogliomas and gangliocytomas represent a distinct subset of neuroepithelial tumors with unique clinical, radiologic, and pathologic features. While generally associated with favorable outcomes, particularly when amenable to complete surgical resection, their biological behavior can vary depending on underlying molecular alterations and anatomical location. Advances in molecular profiling have enhanced our understanding of their pathogenesis, revealing key roles for MAPK/ERK signaling pathway abnormalities. These findings have prompted exploration of targeted therapies in select cases, though their effectiveness and long-term outcomes remain uncertain. Through this review, we have highlighted the current knowledge surrounding these tumors, aiming to support clinicians and researchers in the comprehensive evaluation and management of gangliogliomas and gangliocytomas.

Ethics Statement

Not applicable.

Availability of Data and Material

Data sharing not applicable to this article as no datasets were

generated or analyzed during the study.

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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Lymphomatoid papulosis: a practical review for pathologists

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Lymphomatoid papulosis (LyP) is a primary cutaneous CD30+ lymphoproliferative disorder characterized by a chronic and self-healing recurrent cluster of erythematous papules or nodules on the skin of the trunk and/or extremities. The disease has an indolent clinical course with spontaneous regression or waxing and waning clinical evolution. The histopathologic spectrum of LyP is vast and may show few to numerous atypical cells immersed in a mild to intense inflammatory background. The backbone for the diagnosis is the positivity for CD30, which is one of the criteria to define this group of lymphoproliferative disorders. The association of these different histological and immunophenotypical findings is used to subclassify this disease in different subtypes from A to E, associated with *DUSP22/IRF4* rearrangement, and other rare forms. Although this differentiation is important to raise awareness of different differential diagnosis, it does not impact the prognosis or change the treatment, which is usually centered in symptom relief and faster regression. In this review, we aim to summarize the most updated information of the clinical, histopathological, and molecular characteristics of LyP and provide a practical assessment for the diagnostic features that could help with the main differential diagnosis.

Keywords: Lymphomatoid papulosis; Primary cutaneous ALCL; Anaplastic large cell lymphoma; CD30; *DUSP22*; Cutaneous lymphoma

INTRODUCTION

Lymphomatoid papulosis (LyP) is a chronic and self-healing lymphoproliferative disorder of the skin and is mainly characterized by recurrent and self-healing erythematous papules or nodules on the trunk or extremities [1-8]. This entity is currently characterized under the group of CD30+ lymphoproliferative disorders by both the 5th edition of the World Health Organization of Hematolymphoid Tumors and the International Consensus Classification [2,9]. It was first described in 1968 by Dr. W. Macaulay, who reported a 41-year-old patient with waxing and waning papules for 3 years, for which the biopsy showed an intense infiltrate of atypical cells, suggesting a lymphoma in the skin [10].

Primary cutaneous CD30+ lymphoproliferative disorders include primary cutaneous anaplastic large cell lymphoma (pc-ALCL) and LyP and account for 25% of all cutaneous T-cell lymphomas [5,11]. Although LyP is a relatively frequent condition among the primary cutaneous T-cell lymphomas, this is a very rare condition in the general population and has an incidence of 1.2–1.9 cases per 1 million people [12].

The incidence peak occurs in the fifth decade but can affect all age groups [1,3,13] and it is more common in men than women [1,13,14]. Pediatric cases have been reported and have a similar presentation to the adult ones [15]. Overall, it is also more common in white populations [5] and LyP type A is the most com-

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mon subtype, followed by type D and type B [14], although LyP type B is more common in Hispanics [1].

ETIOPATHOGENESIS AND CLINICAL FEATURES

The etiopathogenesis of LyP is unknown, and no viral etiologic factors, such as Epstein-Barr virus, are associated with the development or progression of the disease. Some cases that show progression to anaplastic large cell lymphoma show resistance to CD30 ligand and alterations involving the TGF- β signaling pathway [5].

Most patients are previously asymptomatic and lack underlying disease, while a subset of cases arises in patients with concurrent or a history of mycosis fungoides (MF) [16]. The typical clinical presentation is characterized by clusters of red or purple papules and small nodules (<2.0 cm each) with different stages of development, spontaneous regression with crusts in a few weeks and an indolent clinical course [1,5,8,11,17,18]. Less commonly, single or multiple crops are observed, and the disease can persist from several weeks to years [1,5,18]. The degree of lesion severity is measured by the number of lesions during the clinical presentation, being defined as mild (<12 lesions), moderate (12–50 lesions), and severe (>50 lesions) [1]. The lesions are more common in the trunk and extremities but can affect any body part [1,3,19] (Fig. 1A). Oral and mucosal involvement is rare [5] and may be accompanied by ulceration and necrosis [20]. The development of secondary lymphomas

may precede, occur concurrently with, or follow the diagnosis and is reported in 10% to 20% of the patients [21,22].

The dermoscopic appearance of these lesions may coexist in different stages of progression and involution [17,23,24], including: (1) pinkish or light brown papules with homogeneous areas (Fig. 1B) and delicate dotted vascular pattern at the periphery; (2) papules with centripetal and tortuous irregular vessels; (3) crusts and papules with central scale and vascular pattern sparing the center in mature lesions; (4) papule with central necrotic ulceration denotes old lesion; (5) postinflammatory pigmentation in lesional areas; and (6) pruritus as the primary symptom in about half of patients [1]; among other more rare patterns.

A correct diagnosis requires an adequate clinical history of papular lesions with spontaneous regression associated with typical histological and immunohistochemical findings [1,3,5,7].

HISTOPATHOLOGICAL FINDINGS

Histopathological presentation in LyP is characterized by a subtle wedge-shaped distribution of the infiltrate, with a wide superficial base and a tapered tip extending into the deep dermis and less frequently into the subcutaneous tissue [25] (Fig. 2A). The wedge-shaped infiltrate may not be readily apparent in a punch biopsy and requires an excisional biopsy for better visualization. The most characteristic appearance is the presence of few to numerous large cells with a Hodgkin or Hodgkin/Reed-Sternberg-like cells mixed with a reactive background

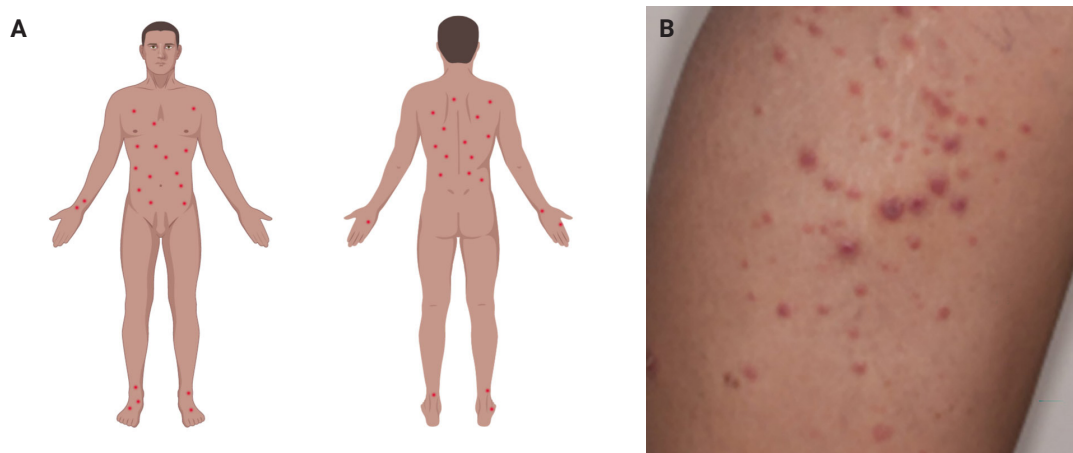


Fig. 1. (A) The classic clinical presentation of lymphomatoid papulosis (LyP) is characterized by clustered erythematous papules mainly distributed on the trunk and extremities (image created with Biorender.com). (B) Clinical image displaying a cluster of macules and papules that are characteristic of LyP. Note that some papules show central crusting, consistent with involuting lesions.

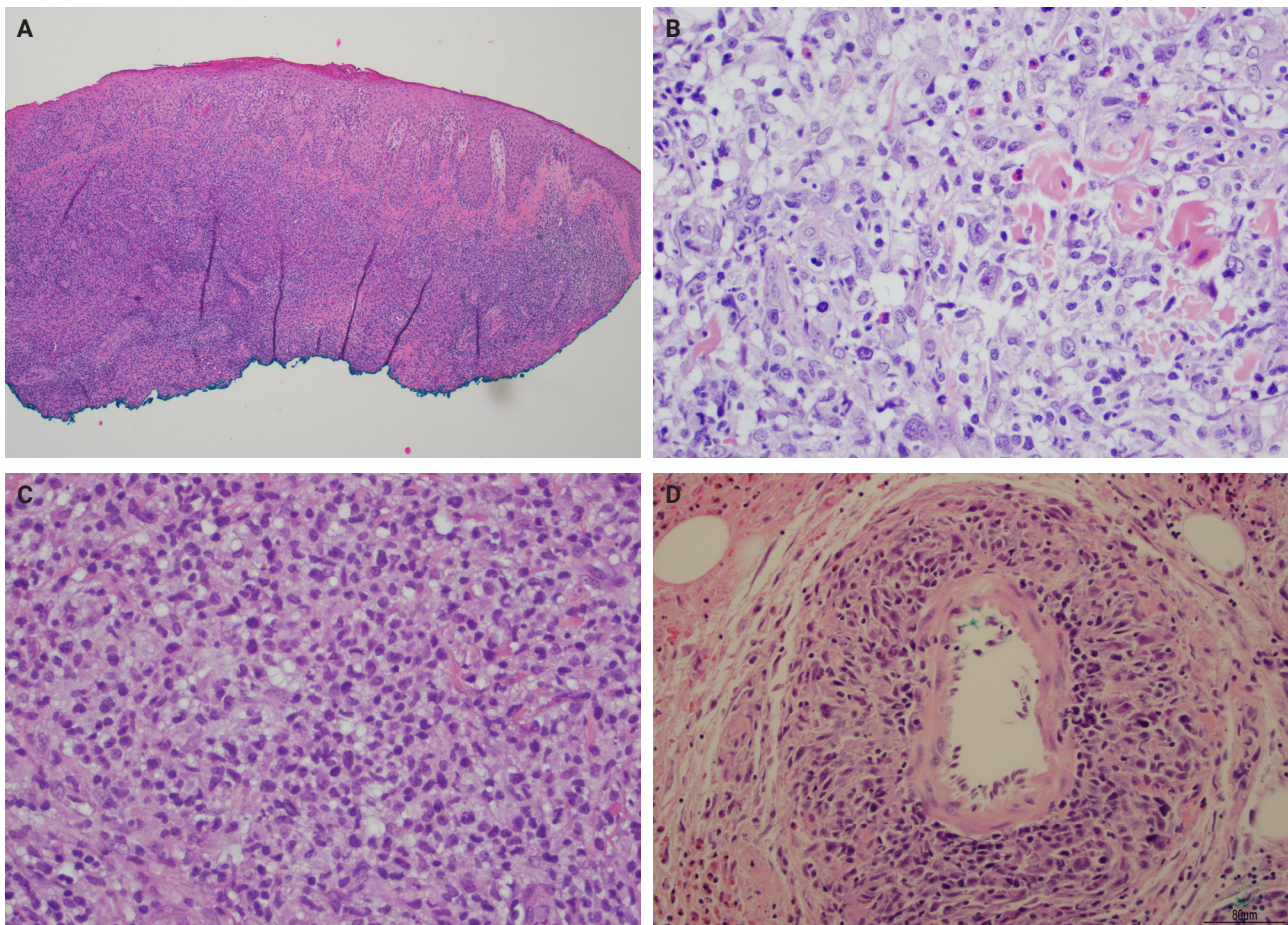


Fig. 2. Lymphomatoid papulosis (Lyp). (A) Histological section of LyP shows a dense and diffuse dermal infiltrate with focal epidermotropism. (B) Histological section of LyP type A shows Hodgkin/Reed-Sternberg-like cells in a background of small lymphocytes, eosinophils, and histiocytes. (C) LyP type C shows a diffuse infiltrate of intermediate to large lymphocytes with moderately abundant cytoplasm, large vesicular nuclei, and distinct nucleoli. (D) LyP type E is characterized by angiotropism as shown in this medium-sized vessel extensively infiltrated by large abnormal lymphocytes.

of small lymphocytes and less frequently eosinophils, plasma cells, and histiocytes [25] (Fig. 2B). Some cases display pseudoepitheliomatous hyperplasia that may raise the possibility of squamous cell carcinoma [26].

The microscopic architecture and immunophenotype of large cells and their background are variable and have led to subclassification of LyP into different categories [1,5,6,13,14,27-33]. In LyP type A, neoplastic CD30+ cells are scattered and admixed with an extensive lymphoid infiltrate of neutrophils, eosinophils, and histiocytes mimicking classic Hodgkin lymphoma (Fig. 2B). In LyP type B, the presentation is characterized by epidermotropism and band-like distribution of small to medium atypical lymphocytes with cerebriform nuclei, usually CD30-negative, and mimicking MF, patch stage. Type C of LyP is composed of

sheets of large neoplastic cells, with or without epidermotropism that display uniform positivity for CD30, mimicking a pc-AL-CL; in this type, only few admixed inflammatory cells are noted. (Fig. 2C). In contrast, LyP type D presents with atypical small to medium-sized lymphocytes with distinct epidermotropism and these neoplastic cells express CD30 and CD8 and mimic pagetoid reticulosis. LyP type E shows an angiocentric and angiodestructive pleomorphic lymphoid infiltrate of small- to medium-sized lymphocytes admixed with scattered large cells uniformly positive for CD30 and the overall appearance is that of an aggressive lymphoma, such as extranodal T/natural killer (NK) cell lymphoma (Fig. 2D). LyP with *DUSP22/IRF4* rearrangement (please note that it is not *DUSP22::IRF4* since there is not a rearrangement between *DUSP22* and *IRF4*; they are genomically

too close for fluorescence in situ hybridization to separate them, so if positive, the rearrangement can be for *DUSP22* or *IRF4*) displays a dense diffuse lymphoid infiltrate (Fig. 3A). A biphasic growth pattern with pagetoid reticulosis-like epidermotropism of small to medium size cerebriform lymphocytes (Fig. 3B) that express dim or lack CD30, while the second component is dermal or periadnexal (Fig. 3C) and the atypical lymphocytes express bright CD30+ (Fig. 3D) [25].

Rare forms of LyP presentation include folliculotropic, syringotropic, granulomatous, and gamma-delta variant. In all cases, large atypical CD30+ cells are identified. In the folliculotropic pattern, there is a perifollicular infiltrate of atypical lymphocytes, cystic dilatation sometimes with rupture of the hair follicle, hyperplasia of the follicular epithelium, intrafollic-

ular pustules (neutrophil collections), and follicular mucinosis [34]. The syringotropic pattern is characterized by eccrine units with periglandular infiltrate [14], while the granulomatous LyP shows mononuclear infiltrate with perivascular, eccrinotropic, and neurotropic distribution associated with non-caseating granulomas [14,35]. The gamma-delta variant may mimic aggressive lymphomas such as primary cutaneous gamma-delta T-cell lymphoma or MF. Most of the cases show cytotoxic immunophenotype; however, the clinical presentation is more akin to LyP [14,36]. Different histologic subtypes may coexist simultaneously or be present in the same patient throughout the clinical evolution of the lesions and even be associated with other CD30+ lymphoproliferative disorders such as pc-ALCL [37].

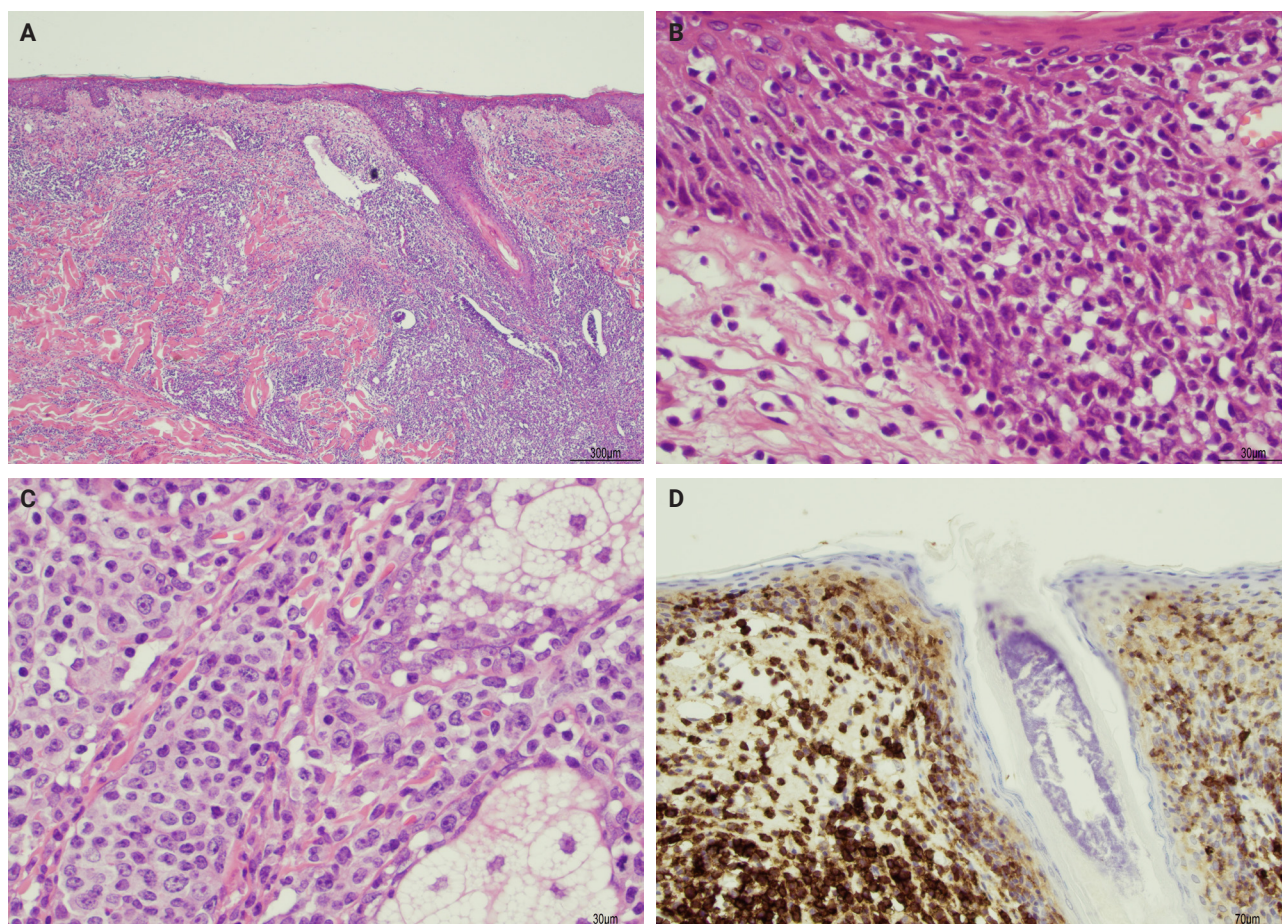


Fig. 3. Lymphomatoid papulosis (LyP) with *DUSP22/IRF4*. (A) Histological section shows dense lymphoid infiltrate in the dermis with epidermotropic infiltrate. (B) High magnification shows a predominance of small epidermotropic lymphocytes. (C) High magnification shows a predominance of large cells in the dermis. (D) Immunohistochemistry for CD30 highlights the biphasic pattern of this LyP variant displaying faint reactivity in epidermotropic lymphocytes while there is strong CD30 reactivity in the dermal lymphocytes. Courtesy of Dr. Sofia A. Garces.

IMMUNOHISTOCHEMICAL AND ANCILLARY STUDIES

The neoplastic cells of LyP usually express CD3, CD4, CD25, CD30, CD45RO, human leukocyte antigen-DR isotype (HLA-DR), T-cell intracellular antigen-1 (TIA1), and granzyme B consistent with an activated T helper immunophenotype. A variable loss of T-cell antigens may be seen. In effect, these cells are characterized by the positivity for CD3 and CD4 as T helper cells. CD45RO defines them as memory/activated T cells while CD25, CD30, and HLA-DR define them as activated lymphocytes. Lastly, TIA1 and granzyme B indicate their cytotoxic phenotype [5,6,24,27-30]. Other T-cell antigens, such as CD2, CD5, and CD7, have a variable expression including complete negativity, depending on the subtype of LyP. Although CD8 is negative in LyP types A, B, and C, it is usually positive in LyP type D and has a variable positivity in type E and *DUSP22/IRF4* rearrangement cases. The biomarker scenario of LyP is mainly characterized by the CD30 expression in the neoplastic cells, helping not only in the diagnostic definition of the entity, but also with the possibilities of new therapeutic options. Table 1 summarizes the main immunophenotypical features of different subtypes of LyP.

Monoclonal rearrangements of the T-cell receptor gamma (*TRG*) or beta (*TRB*) chains are usually detected [5] and may overlap with other coexisting lymphomas, such as MF and pc-ALCL [38]. Recurrent mutations in epigenetic modifying genes (*SETD2*, *KMT2A*, *KMT2D*, and *CREBBP*) are the most frequently seen, but no specific mutations are observed, since a similar mutational profile is seen in pc-ALCL [39]. Recurrent *NPM1::TYK2* gene fusions in LyP induce activation of the STAT signaling pathway [40,41]. More recently, cases with characteristic *DUSP22/IRF4* rearrangement/6p25.3 translocation cases have been described and show characteristic features, such as common presentation in older patients, localized lesions with clinical presentation suggesting benign inflammatory dermatoses or epithelial tumors, and histological presentation with a bimodal pattern. The epidermotropic lymphocytes are small and are negative or express weak CD30, while the dermal cells are large, express strong CD30 and lymphocyte enhancer binding-factor 1, and are negative for TIA1 [6].

DIFFERENTIAL DIAGNOSIS

The differential diagnosis of LyP is broad due to the great overlap in the clinical, histological, and immunophenotypical

Table 1. Immunohistochemical summary of LyP types

Type of LyP	CD2	CD3	CD4	CD5	CD7	CD8	CD30	ALK1	Other markers
Type A	+/-	+	+	+/-	+/-	-	+	-	TIA1+
Type B	+/-	+	+	+/-	+/-	-	-	-	TIA1+ (expression variable)
Type C	+/-	+	+	+/-	+/-	-	+	-	TIA1+
Type D	+	+	-	-	-	+	+	-	TIA1+ βF1+ CD56- EBER-
Type E	+	+	-/+	+	+/-	+	+	-	CD45RA- CD45RO+/- TIA1+ βF1+ EBER-
LyP with <i>DUSP22/IRF4</i> rearrangement	+/-	+	-	+/-	+/-	+/-	+	-	CD15+, LEF1+ TIA1- Ki67 (>80%) TCRβ+
Folliculotropic LyP	+	+	+	+/-	-	-	+	-	-
Granulomatous LyP	+	+	+	+	-	-	+	-	-

LyP, lymphomatoid papulosis; ALK1, anaplastic lymphoma kinase 1; TIA1, T-cell intracellular antigen 1; EBER, Epstein-Barr virus-encoded small RNA; LEF1, lymphoid enhancer-binding factor 1; TCRβ, T-cell receptor beta chain.

presentations with malignant skin diseases as well as benign dermatoses. The main differential diagnoses according to the specific type of LyP are summarized in [Tables 2 and 3](#).

Primary cutaneous anaplastic large cell lymphoma (pc-ALCL)

Pc-ALCL is the main differential diagnosis of LyP type A and type C [27,42]. Both LyP and pc-ALCL are more prevalent in young adults. In LyP, the clinical presentation is characterized by multiple papules, papulonodular or nodular lesions <2 cm, while in pc-ALCL localized or single nodules or papules more than 2 cm are more common. The histological presentation in pc-ALCL is characterized by diffuse and homogeneous dermal infiltration of anaplastic cells without epidermotropism, while in LyP could vary from polymorphous and wedge-shaped in LyP type A and sheets of large lymphocytes with or without epidermotropism with few admixed inflammatory cells in type

C. Cases of LyP type C could be morphologically identical to pc-ALCL, and because of this, the clinical correlation is fundamental for the final diagnosis. Both entities have a T-helper immunophenotype, characterized by CD3+ and CD4+, negativity for CD8, and diffuse positivity for CD30. Cases of pc-ALCL can also harbor DUSP22/IRF4 gene rearrangements, while cases of LyP usually express multiple myeloma oncogene 1/IRF4 and are negative for anaplastic lymphoma kinase-1, being additional markers that could help in the differential diagnosis [43,44]. The outcome is excellent in both entities [27,42].

Early-stage MF

Cases of LyP type B could have some overlapping histological and immunophenotypical presentation with early-stage MF. Both neoplasms present in adults and have a slight predominance in males. The clinical presentation in LyP is characterized by papular, papulonodular, or nodular lesions, while ear-

Table 2. Main clinical features of differential diagnoses of different subtypes of LyP

	Age	Sex (M:F)	Clinical feature	Treatment	Outcome
LyP type A	Fifth decade	2–3:1	Papular, papulonodular or nodular; <2 cm	None for spontaneous regression; symptomatic treatment for relief	Excellent
Primary cutaneous anaplastic large cell lymphoma	Seventh decade	2–3:1	Localized nodule or papule; usually >2 cm	Excision, radiation, or chemotherapy, alone or combined	Excellent
LyP type B	Fifth decade	2–3:1	Papular, papulonodular, or nodular	Spontaneous regression or symptomatic treatment	Excellent
Early-stage mycosis fungoides	Sixth decade	2:1	Patches and plaques	Ultraviolet light	Good
LyP type C	Fifth decade	2–3:1	Papular, papulonodular, or nodular; <2 cm	Spontaneous regression or symptomatic treatment	Excellent
Primary cutaneous anaplastic large cell lymphoma	Seventh decade	2–3:1	Localized nodule or papule; >2 cm	Radiation or chemotherapy	Good
LyP type D	Third decade	2:1	Clustered papules or small nodules	Spontaneous regression or symptomatic treatment	Excellent
Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma	Fourth decade	5:2	Ulcerative patches or plaques	Chemotherapy, stem cell transplant	Poor
LyP type E	Sixth decade	3:1	Recurrent clustered papular lesions with rapid progression to hemorrhagic necrotic ulcers	Spontaneous regression or symptomatic treatment	Excellent
Extranodal NK/T-cell lymphoma	Fifth and sixth decades	2:1	Multiple necrotic lesions	Progressive disease	Poor
LyP with DUSP22/IRF4 rearrangement	Eighth decade	4.5:1	1 or multiple eruptive and papulonodular lesions in a single body area	Spontaneous regression or symptomatic treatment	Excellent
Transformed mycosis fungoides	Seventh decade	1.1:1	Patches, plaques, and tumor	Radiation, chemotherapy	Poor
Folliculotropic LyP	Sixth decade	4.5:1	Follicular pustules	Spontaneous regression	Excellent
Folliculitis	Variable	Variable	Superficial folliculitis	Topical treatment	Excellent
Granulomatous LyP	Fifth decade	5:4	Nodules	Spontaneous regression	Excellent
Discoid lupus erythematosus	Fifth decade	1:2	Discoid lesions	Systemic agents, phototherapy	Excellent

LyP, lymphomatoid papulosis.

Table 3. Main pathologic and immunophenotypic features of differential diagnoses of different subtypes of LyP

	Microscopy	Immunophenotype
LyP type A	Wedge-shaped (subtle) with scattered Hodgkin/Reed-Sternberg-like cells admixed with neutrophils, eosinophils, and histiocytes	CD3+, CD4+, CD8–, CD30+
Primary cutaneous anaplastic large cell lymphoma	Diffuse and homogeneous dermal infiltrate of anaplastic large cells	CD3 (variable), CD4+, CD8–, CD30+
LyP type B	Epidermotropism and band-like distribution of small/medium atypical lymphocytes with cerebriform nuclei (MF-like)	CD4+, CD8–, CD30–
Early-stage MF	Cerebriform cells with epidermotropism predominate along the basal layer of epidermis	CD4+, CD7–, CD8–, CD30–
LyP type C	Sheets of large lymphocytes with or without epidermotropism and few admixed inflammatory cells	CD4+, CD8–, CD30+ (uneven)
Primary cutaneous anaplastic large cell lymphoma	Diffuse dermal infiltrate of large anaplastic lymphocytes	CD3 (variable), CD4+, CD8–, CD30+ (uniform)
LyP type D	Epidermotropism with pagetoid reticulis-like	CD2+, CD3+, CD4–, CD8+, CD30+, TIA1+
Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma	Small to intermediate-sized pleomorphic lymphocytes with epidermotropism	CD2–, CD3+, CD7+, CD8+, CD30–, CD56–
LyP type E	Angiocentric and angiodestructive pleomorphic lymphoid infiltrate of small/medium size	CD2+, CD3+, CD4–/+, CD8+, CD30+, TIA1+, EBV–
Extranodal NK/T-cell lymphoma	Atypical lymphocytes; angiocentric and angiodestructive in most cases	CD3–, CD4–, CD5–, CD8–/+, CD30+, CD56+, TIA1+, EBER+
LyP with <i>DUSP22/IRF4</i> rearrangement	Biphasic growth pattern: epidermotropic small cells, versus dermal or periannexal medium to large cells	CD3+, CD4–, CD8 (variable), LEF1+, TIA1–
Transformed MF	Cerebriform with increased large cells in the dermis	CD3+, CD4+, CD7–, CD8–, CD30 (variable)
Folliculotropic LyP	Perifollicular infiltrate of atypical lymphocytes and follicular mucinosis	CD3+, CD4+, CD30+
Folliculitis	Moderate mixed inflammatory cells in the follicular ostium	Polyclonal
Granulomatous LyP	Noncaseating granulomas associated with mononuclear infiltrate with perivascular, eccrinotropic, and neurotropic distribution	CD3+, CD4+, CD5–, CD7–, CD8–, CD30+
Discoid lupus erythematosus	Edema and a mild infiltrate of inflammatory cells (lymphocytes) in the upper dermis	CD3+, CD4+; no loss of T-cell antigens

LyP, lymphomatoid papulosis; MF, mycosis fungoides; TIA1, T-cell intracellular antigen-1; NK, natural killer; EBV, Epstein-Barr virus; EBER, Epstein-Barr virus-encoded small RNA.

ly-stage MF presents with patches and plaques. Histologically, an epidermotropic infiltrate of small/medium cerebriform cells is observed in both entities and might be indistinguishable. The immunophenotype is also very similar in both, being CD3+, CD4+, CD8–, and CD30–. The clinical correlation is fundamental to precisely determine the diagnosis [27,45].

Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma

This is a rare and clinically aggressive primary cutaneous lymphoma with a poor prognosis, which may have overlapping features with LyP type D. Both affect young adult men; however, the clinical presentation of primary cutaneous CD8+ aggressive

epidermotropic cytotoxic T-cell lymphoma (PC CD8-AECTCL) is more dramatic and the lesions are usually ulcerative patches or plaques with rapid progression and necrosis. The main immunophenotypic difference is centered on the CD30 expression, which is positive in LyP and negative in PC CD8-AECTCL; however, the cytotoxic immunophenotype (CD3+CD4–CD8+) is common in both [30,46].

Extranodal NK/T-cell lymphoma involving skin

LyP type E is the main differential diagnosis of extranodal NK/T-cell lymphoma (ENKTCL). Both affect more men in the fifth to sixth decade of life. While LyP type E presents with recurrent clustered papular lesions with rapid progression to hemorrhagic

ic necrotic ulcers, ENKTCL presents with multiple necrotic lesions. The histological presentation can be identical in both, characterized by angiocentric and angiodestructive pleomorphic lymphoid infiltrate of small to medium size and significant inflammatory background. The main immunophenotypical difference is centered on the association with Epstein-Barr virus, which is present in ENKTCL and negative in LyP type E. ENKTCL is also very aggressive and has a bad prognosis [6,27,47].

Large cell transformation of MF

Both LyP with *DUSP22/IRF4* rearrangement and large cell transformation of MF affect elderly men. LyP with *DUSP22/IRF4* presents with one or multiple eruptive and papulonodular lesions in a single body area, while patients with large cell transformation of MF refer to a history of evolution of patches, plaques, and tumors. This LyP subtype usually displays a biphasic growth pattern, where the epidermotropic component is that of small cells and the dermal/periadnexal component is composed of medium to large cells. Large cell transformation of MF has sheets of large cells, occasionally admixed with cerebriform lymphocytes. The immunophenotype is similar in both entities and CD30 tends to be more uniform in cases of LyP, but a diffuse and strong positivity cannot exclude large cell transformation of MF. The clinical correlation is fundamental to rendering the final diagnosis [6,48,49].

Additional differential diagnosis

Due to the broad histological and clinical manifestation of LyP, other entities that could also be considered as differential diagnosis, but will not be contemplated in this review, are (1) pityriasis lichenoides/pityriasis lichenoides et varioliformis acuta; (2) arthropod bite reaction; (3) reactive CD30+ cutaneous infiltrates; (4) systemic ALCL involving skin; and (5) classic Hodgkin lymphoma involving skin.

TREATMENT AND PROGNOSTIC FACTORS

Most patients do not require specific treatment [1,6,7,18], and clinical observation only is enough. Most of the treatments are aimed at symptom relief and faster regression. There are several treatment modalities, such as (1) low-dose methotrexate [11,18,31]; (2) psoralen ultraviolet A [1,7,11,18], which is related to longer disease-free survival: 10–36 months [18]; (3) topical corticosteroids [1,7,18]; (4) brentuximab vedotin, an active

and well tolerated option and has shown a very high response rate in some reports [1,4]; (5) bexarotene [1]; (6) topical ruxolitinib [41]; and (7) topical alkylating agents (e.g., chlormethine gel) [50]. Treatment discontinuation often led to relapses and therapies often suppress lesions without altering the long-term disease course. Since the clinical course and the low necessity of treatment, few innovative therapeutic options are studied in clinical trials (<https://clinicaltrials.gov/search?cond=Lymphomatoid%20Papulosis&viewType=Card>).

The prognosis is excellent [5,7,11,13], and only around 5% of the patients die of disease over a median period of 4 years, particularly patients with established underlying cutaneous lymphoma [1]. Although it is important to differentiate different subtypes of LyP for a proper differential diagnosis, the histologic types do not have prognostic significance [27].

Patients with a diagnosis of LyP are recommended clinical follow-up, since there is a risk of developing a second lymphoid malignancy (e.g., MF and Hodgkin lymphoma) [4,5,7,11,13,18]. Patients with monoclonal *TRB* or *TRG* gene rearrangements or a mixed type of LyP have a higher risk of progressing to lymphoma [13], and an increased risk of subsequent lymphoma has been reported in men and in patients with LyP types B and C, while LyP type A and D are less frequently associated with lymphoma development [1]. Cases of LyP type A are the most prevalent subtype associated with early relapses [18] and elevated serum lactate dehydrogenase is associated with a high risk of associated lymphoproliferative disorders in all cases of LyP [8].

Ethics Statement

Not applicable.

Availability of Data and Material

Data sharing not applicable to this article as no datasets were generated or analyzed during the study.

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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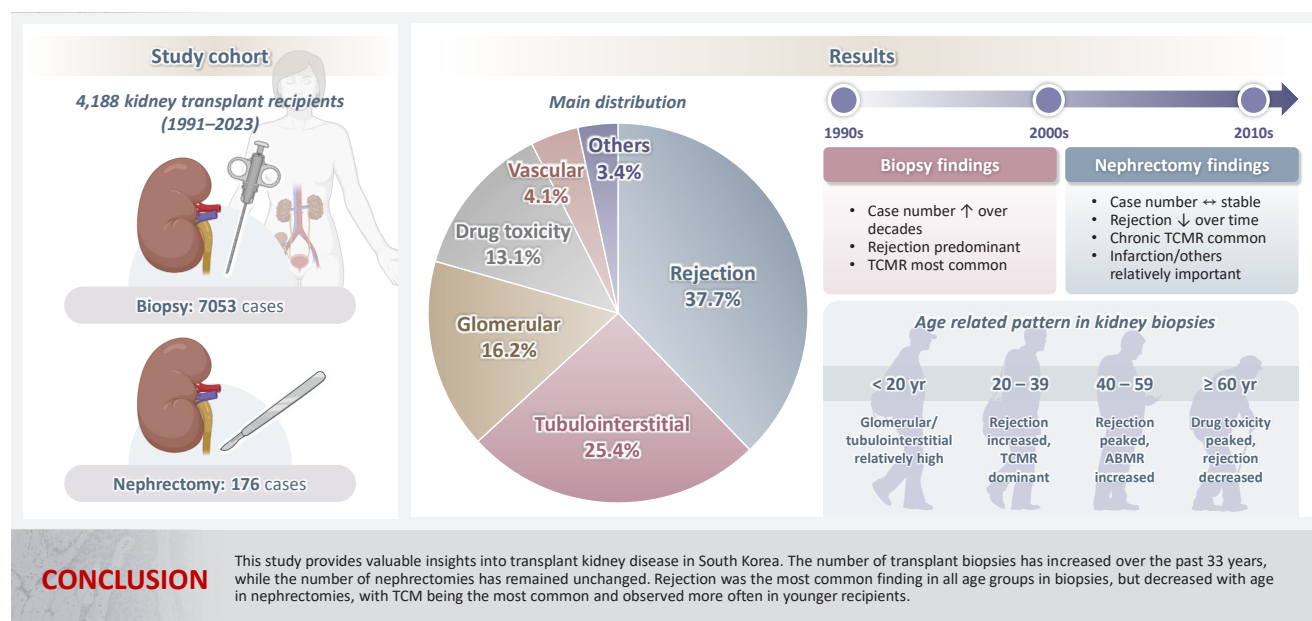
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A single-institution demographic study of pathologically proven renal disease in kidney transplant recipients over the last 33 years

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Graphical abstract



A single-institution demographic study of pathologically proven renal disease in kidney transplant recipients over the last 33 years

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Background: While the number of kidney transplants for end-stage renal disease (ESRD) is increasing, studies examining the long-term demographic analyses based on pathological diagnosis of transplant kidney remain limited. **Methods:** We conducted a retrospective analysis of 4,188 transplant recipients who underwent either biopsy or nephrectomy from 1991 to 2023 at Seoul St. Mary's Hospital. **Results:** Among 7,229 pathologically confirmed cases, rejection was the most prevalent (37.7%), followed by tubulointerstitial (25.4%), glomerular, drug toxicity, and vascular diseases. In 7,053 transplant biopsies, rejection was predominant across all age groups, with T-cell mediated (TCM) category being the most common (60.1%), followed by antibody-mediated and mixed. Drug toxicity increased with age ($p = .047$), while glomerular and tubulointerstitial diseases were highest in recipients under 20 ($p < .001$). Among glomerular diseases, IgA-related glomerulonephritis (45.2%) was the most common. In 176 transplant nephrectomies, the most common diagnosis was rejection (33.5%), followed by renal infarction (19.9%), tubulointerstitial, vascular, glomerular disease, and drug toxicity. "Others" included infarction, ESRD, and lymphangiectasia, which increased with age ($p = .011$). In nephrectomy cases, rejection decreased over time, with chronic TCM rejection (40.7%) being the most frequent. **Conclusions:** This study provides valuable insights into transplant kidney disease in South Korea. The number of transplant biopsies has increased over the past 33 years, while the number of nephrectomies has remained unchanged. Rejection was the most common finding in all age groups in biopsies, but decreased with age in nephrectomies, with TCM being the most common and observed more often in younger recipients.

Keywords: Biopsy; Kidney; Nephrectomy; Pathology; Transplantation

INTRODUCTION

Kidney transplantation (KT) is the main treatment for end-stage renal disease (ESRD), but maintaining the transplanted kidney remains a challenge [1,2]. Biopsies are routinely performed to diagnose and manage various conditions affecting transplanted kidneys, and if the recipient's condition worsens, transplant nephrectomy is performed [2]. According to the Global Burden of Disease, Injuries, and Risk Factor Study, 697.5 million people had chronic kidney disease in 2017, which corresponds to a global prevalence of 9.1%, and resulted in 1.2 million deaths [3]. Furthermore, as of 2016, Korea recorded the sixth-highest

incidence of ESRD worldwide, exceeding 300 cases per million [4], and this trend is particularly pronounced due to the aging population and the increase in diabetic nephropathy (DMN) [5]. Since the first KT in Korea was performed in 1969 [6], there has been a steady rise in the number of transplant recipients [7]. Numerous studies have explored the clinical outcomes of KT, examining success and failure rates and their causes [8-12], and to determine the reasons for kidney transplant losses [13-20]. However, few studies have conducted long-term demographic analyses based on pathological diagnoses [21,22].

In this study, we retrospectively reviewed all transplant kidney diseases performed over a 33-year period at Seoul St. Mary's Hos-

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pital in South Korea, to conduct a long-term demographic analysis based on the pathological diagnosis of transplant kidneys.

MATERIALS AND METHODS

Recipient population

Our retrospective investigation encompassed 4,188 recipients who underwent pathology examinations of transplanted kidneys at Seoul St. Mary's Hospital from January 1991 to December 2023. We reviewed pathological and clinical records of the recipients. Exclusion criteria included incomplete medical records, inadequate tissue specimens, frozen biopsy specimens, and donor/recipient renal artery biopsy specimens.

This study aimed to investigate renal inflammatory diseases in transplanted kidneys, excluding tumors. We collected the data on: kidney specimen type (either biopsy or surgical), date of procedure, recipient age, sex, transplantation status and histopathologic diagnosis. This study considered each tissue (biopsy or nephrectomy) separately. That is, if multiple pathological diagnoses were present in a single tissue, these diagnoses were counted separately. If multiple diagnoses from a single transplant kidney biopsy or nephrectomy specimen were received, the one that appeared to be the primary cause of renal allograft dysfunction prompting the diagnostic procedure was designated as the major diagnosis. This was used to analyze correlations between the recipient's sex, age, time of diagnosis, and the disease. Other diagnoses were counted as minor diagnoses. All major and minor diagnoses were used to analyze the frequency and distribution of each disease. Each case was diagnosed according to the pathological diagnostic entity, and then was classified based on disease type, demographic data, and changes over time.

Data source

Our study included both biopsy and nephrectomy specimens to ensure comprehensive analysis of entire renal diseases in transplant kidneys. We analyzed them separately and classified into the following six disease types based on pathologic diagnoses: Rejection, drug toxicity, glomerular disease, tubulointerstitial disease, vascular disease, and "Others" (diagnoses not fitting clearly into the previous five types).

The pathologic classification of rejection in transplanted kidneys was based on the 1997 Banff classification [23], incorporating updates from the Banff 2019 [24] and Banff 2022 [25] kidney meeting reports. Rejection was classified into T-cell mediated (TCM), antibody-mediated (ABM), and mixed cat-

egories. TCM included three categories: borderline change, acute T-cell mediated rejection (TCMR), and chronic TCMR. ABM included hyperacute rejection, acute antibody-mediated rejection (ABMR), chronic ABMR, microvascular inflammation (MVI), donor-specific antibody (DSA) negative and C4d negative, acute/chronic transplant glomerulopathy, and C4d staining without evidence of rejection. Mixed category included cases in which diagnoses from both of the aforementioned categories were simultaneously presented in one transplant recipient. All recipients diagnosed with drug toxicity were those who used immunosuppressants, specifically calcineurin inhibitors such as cyclosporine A and FK506.

Glomerular disease was classified according to the following three criteria: first, classification according to pathological diagnostic entity; second, classification according to morphologic category; third, classification according to etiologic category. According to the morphologic category, glomerular disease was classified into proliferative, non-proliferative, sclerosing, mixed (diseases with two or more of the previous categories), and other (diseases not fitting into the previous categories). According to the etiologic category, glomerular disease was classified into primary, secondary, hereditary, mixed (diseases with both primary and secondary features), and unknown (diseases not fitting into the previous categories).

Tubulointerstitial and vascular diseases were classified according to the pathological diagnostic entity. All cases were analyzed across three consecutive decades (1991–2000, 2001–2010, and 2011–2020). To examine correlations between recipient age and disease types, participants were stratified into four age groups (under 20 years, 20–39 years, 40–59 years, and over 60 years).

Statistical analyses

Continuous variables were presented as mean $\pm$ standard deviation and compared using ANOVA or the Kruskal-Wallis test. When significant differences were observed, Tukey's honestly significant difference test and pairwise comparisons of proportions with Bonferroni correction were performed for post-hoc analysis. Categorical variables were reported as frequency (%) and compared using the chi-squared test. For the analysis of the trend in disease prevalence over the time period, linear regression analysis was used.

RESULTS

Renal disease in transplant kidney

A total of 4,188 recipients with renal disease in transplant kidneys (4,072 biopsies and 116 nephrectomies) were enrolled. The sex ratio was 1.60:1 (Fig. 1A), and the mean age was 44.7 ± 12.2 years. In total, 7,229 major and minor renal diagnoses were made in all recipients by biopsy or nephrectomy. Among them, rejection was diagnosed in 2,723 cases (37.7%), showing the highest frequency, followed by tubulointerstitial (1,837 cases, 25.4%), glomerular (1,174 cases, 16.2%), drug toxicity (949 cases, 13.1%), and vascular diseases (295 cases, 4.1%) (Fig. 1B). When the mean age and sex ratio of the recipients who underwent biopsy or nephrectomy were compared in Table 1, no significant differences were observed between the two groups in terms of age and sex ratio.

Renal disease in transplant kidney biopsy

The sex ratio of 4,072 recipients who underwent renal biopsy was 1.61:1, and the mean age was 44.7 ± 12.2 years (Table 1). The incidence of renal diseases diagnosed through biopsy began to increase in the 20s, peaked in the 40s, and then gradually

decreased until the 60s (Fig. 2A). A total of 2,072 recipients were diagnosed with rejection. The number started to increase in their 10s, 20s, and 30s, peaked in their 40s, and decreased in the 50s, 60s, and over 70s. The number of recipients diagnosed with drug toxicity was 337, and it began to increase in their 10s, 20s, 30s, 40s, reached a peak in their 50s, and declined in 60s, and over 70s. The number of glomerular disease recipients was 707, and it began to increase in their 10s, 20s, and 30s, peaked in their 40s, and then decreased in 50s, 60s, and over 70s. Tubulointerstitial diseases were diagnosed in 701 recipients, and the incidence started to increase in their 10s, 20s, and 30s, peaked in their 40s, and decreased in 50s, 60s, and over 70s. Vascular disease was diagnosed in 76 recipients, with the highest prevalence in their 30s, 40s, and 50s, followed by those in their 60s, 20s, 70s or older, and teenagers.

Trends of renal disease in transplant kidney biopsy

Decadal changes of biopsies performed for transplant renal disease

The number of transplant biopsies increased 2.19-fold over three decades from the 1990s (846 recipients) to the 2000s (1,007

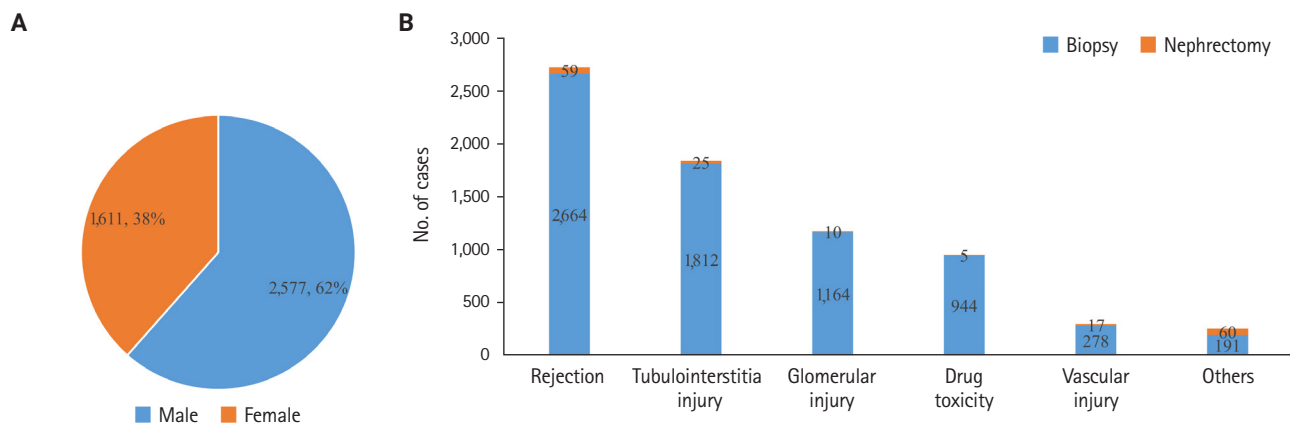


Fig. 1. (A) Sex ratio of 4,188 patients with transplant kidney disease including biopsy and nephrectomy. (B) Diagnostic categories of kidney disease in transplant biopsy and nephrectomy cases.

Table 1. Demographic data of 4,188 patients with renal disease in transplant kidney

Category	All samples	Kidney biopsy	Nephrectomy	p-value
No. of patients	4,188 (100)	4,072 (97.2)	116 (2.8)	-
Major and minor diagnoses	7,229 (100)	7,053 (97.6)	176 (2.4)	-
Age (yr)	44.7 ± 12.2	44.7 ± 12.2	46.1 ± 11.9	.223
Male:female ratio	1.60:1	1.61:1	1.42:1	.579

Values are presented as number (%) or mean $\pm$ standard deviation.

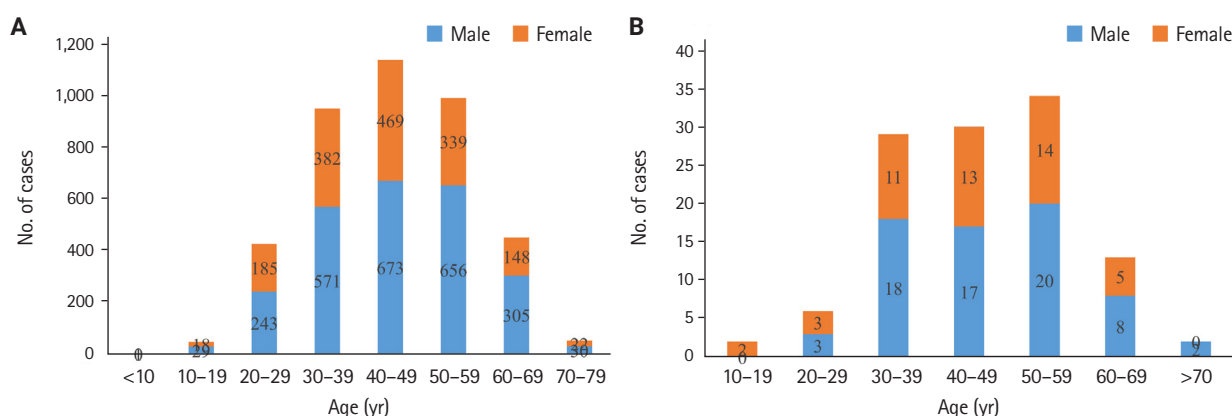


Fig. 2. (A) Age and sex distribution of 4,072 patients who underwent biopsy for transplant kidney disease. (B) Age and sex distribution of 116 patients who underwent nephrectomy for transplant kidney disease.

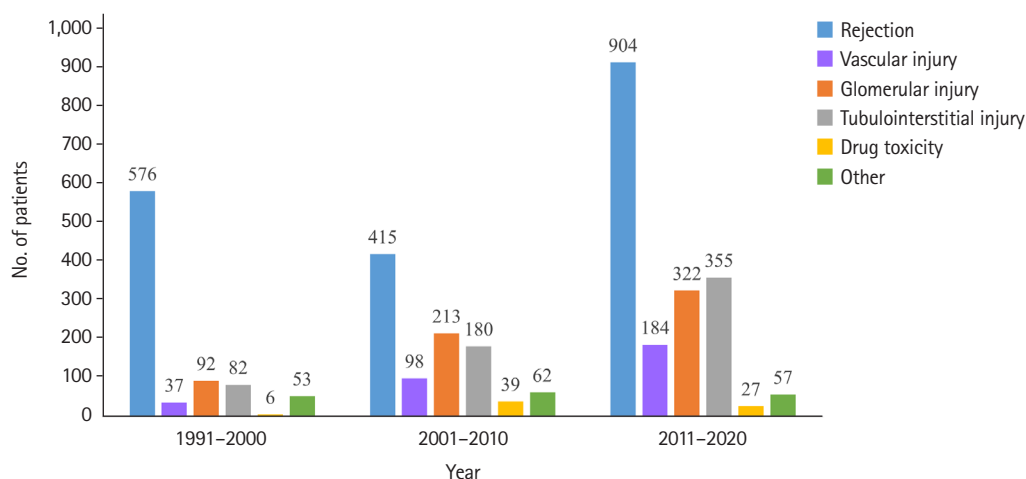


Fig. 3. Trends over three decades in the distribution of renal disease types in 3,702 transplant biopsy patients. They were analyzed for three consecutive decades (1991-2000, 2001-2010, and 2011-2020).

recipients) and the 2010s (1,849 recipients).

Decadal changes of transplant biopsies classified by renal disease types

Rejection rates decreased from the 1990s (576 recipients, 68.1%) to the 2000s (415 recipients, 41.2%), but they increased in the 2010s (904 recipients, 48.9%). Except for rejection, the number and frequency of other renal diseases increased from the 1990s to the 2000s and 2010s (Fig. 3). The changes in the proportions of each disease types were as follows: drug toxicity - 1990s (37 recipients, 4.4%), 2000s (98 recipients, 9.7%), and 2010s (184 recipients, 10.0%); tubulointerstitial - 1990s (82 recipients, 9.7%), 2000s (180 recipients, 17.9%), and 2010s (355 recipients, 19.2%);

glomerular - 1990s (92 recipients, 10.9%), 2000s (213 recipients, 21.2%), and 2010s (322 recipients, 17.4%). No consistent change was observed in vascular diseases over time: 1990s (6 cases, 0.7%), 2000s (39 cases, 3.9%), and 2010s (27 cases, 1.5%).

Differences in transplant renal disease types by age groups

The distribution of renal disease was classified by age group (Fig. 4). Rejection represented the highest proportion in all age groups, reaching its peak frequency of 52.6% in their 40s and 50s. The proportion of drug toxicity increased with age, peaking in those over 60 years (11.9%) ($p = .047$). In contrast, glomerular (25.0%) and tubulointerstitial diseases (22.9%) accounted for the highest proportion in recipients under 20 years ($p < .001$).

Furthermore, an analysis of age distribution across rejection categories (Fig. 5) revealed that TCM (42.0 ± 11.6 years) was more common in younger recipients compared to ABM (47.5 ± 11.0 years) and mixed (48.1 ± 11.7 years) categories ($p < .001$).

When the glomerular morphologic categories were analyzed by age (Fig. 6A), proliferative (mean age, 44.3 ± 11.2 years) and non-proliferative (46.2 ± 13.9 years) glomerulonephritis (GN) were found in younger recipients, whereas sclerosing (51.4 ± 12.8 years) and mixed (51 ± 11.3 years) GN were found in older recipients ($p = .001$). In an analysis by etiological categories (Fig. 6B), the mean ages were as follows: primary (43.9 ± 11.9 years), secondary (53.5 ± 12.4 years), hereditary (47 ± 9.42 years), and mixed GN (52.3 ± 12.8 years). Recipients with primary GN were notably younger at the time of diagnosis ($p < .001$).

Demographics and pathologic diagnostic entities in transplant biopsy

Demographic findings on renal diseases diagnosed through biopsy are shown in Table 2. A total of 7,053 cases were diagnosed in 4,072 recipients. Rejection was the most prevalent (2,664 cases, 37.8%), followed by tubulointerstitial (1,812 cases, 25.7%), glomerular (1,164 cases, 16.5%), drug toxicity (944 cases,

13.4%), and vascular diseases (278 cases, 3.9%). There was a significant variation in mean age by disease type ($p = .007$), primarily due to the significantly lower mean age of patients in the "Others" category. Post-hoc Tukey analysis revealed that patients in the "Others" category were significantly younger than those in the drug toxicity, glomerular disease, and tubulointerstitial disease groups (all adjusted $p < .01$). After excluding the "Others" category, only the rejection and drug toxicity groups showed a significant age difference (adjusted $p = .024$).

When observing the frequency of occurrence by sex, drug toxicity was observed to have a slightly higher proportion of men compared to other groups, indicating a significant difference among disease types ($p = .002$). Post-hoc pairwise comparisons with Bonferroni correction revealed a significant difference in sex distribution only between the rejection and drug toxicity groups. This remained true both in the analysis including "Others" categories (adjusted $p = .003$) and in the analysis excluding "Others" categories (adjusted $p = .002$). No other comparisons showed statistically significant differences.

Pathologic diagnostic entities of rejection in transplant biopsy

Rejection was categorized based on the Banff classification, with 2,664 cases diagnosed in 2,072 recipients (Table 3). The TCM category (1,602 cases, 60.1%) was the most frequently diagnosed, followed by ABM (629 cases, 23.6%) and mixed categories (433

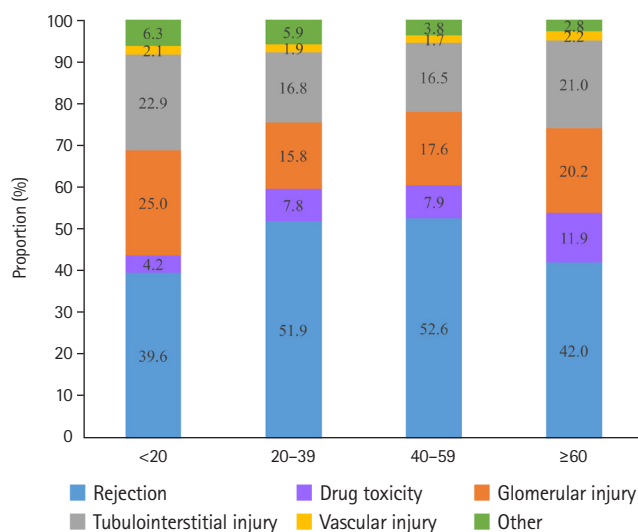


Fig. 4. Proportional analysis of renal disease types among four age groups in transplant kidney biopsies. Patients were stratified into four age groups; under 20 years, 20–39 years, 40–59 years, and over 60 years. The proportion of drug toxicity increased with age, peaking in those over 60 years ($p = .047$). Glomerular and tubulointerstitial diseases accounted for the highest proportions in recipients under 20 years ($p < .001$).

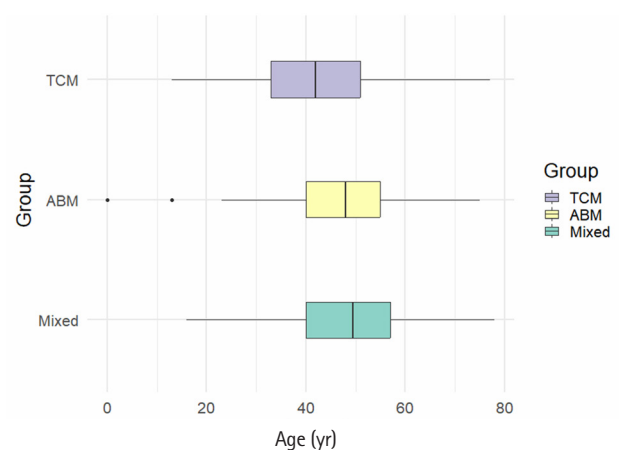


Fig. 5. Box plot analysis of age distribution according to rejection categories in transplant kidney biopsy. The mean age and standard deviation of each group was as follows: T-cell mediated (TCM) (42.0 ± 11.6 years), antibody mediated (ABM) (47.5 ± 11.0 years), and mixed (48.1 ± 11.7 years) ($p < .001$).

Table 2. Demographic data of 4,072 patients with renal disease in transplant kidney biopsy

Category	All diseases	Rejection	Drug toxicity	Glomerular disease	Tubulointerstitial disease	Vascular disease	Others	p-value (excluding others)	p-value (including others)
No. of patients (%)	4,072 (100)	2,072 (50.9)	337 (8.3)	707 (17.4)	701 (17.2)	76 (1.9)	179 (4.4)	-	-
Major and minor diagnosis, cases (%)	7,053 (100)	2,664 (37.8)	944 (13.4)	1,164 (16.5)	1,812 (25.7)	278 (3.9)	191 (2.7)	-	-
Age (yr)	44.7 ± 12.2	44.2 ± 11.9	46.3 ± 12.5	45.5 ± 12.4	45.3 ± 12.8	45.6 ± 12.9	41.6 ± 11.9	.007	<.001
Male:female ratio	1.61:1	1.43:1	2.30:1	1.71:1	1.69:1	1.92:1	1.89:1	.002	.002

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

Table 3. Classification of 2,664 transplant biopsies according to the rejection and diagnostic categories

Major and minor diagnosis	No. of cases	Total (%)	Subtotal (%)
T-cell mediated	1,602	60.1	100
Borderline change	914	34.3	57.1
Acute TCMR	567	21.3	35.4
Chronic TCMR	121	4.5	7.6
Antibody mediated	629	23.6	100
Hyperacute rejection	14	0.5	2.2
Acute ABMR	138	5.2	21.9
Chronic ABMR	125	4.7	19.9
MVI, DSA negative and C4d negative	167	6.3	26.6
Acute/chronic transplant glomerulopathy	115	4.3	18.3
C4d staining without evidence of rejection	70	2.6	11.1
Mixed	433	16.2	100
MVI and TCMR	133	5.0	30.7
MVI and borderline change	123	4.6	28.4
ABMR and TCMR	94	3.5	21.7
ABMR and borderline change	83	3.1	19.2
Total	2,664	100	-

TCMR, T-cell mediated rejection; ABMR, antibody mediated rejection; MVI, microvascular inflammation; DSA, donor specific antibody.

cases, 16.2%). Borderline change was the most predominant at 34.3%, followed by acute (21.3%) and chronic (4.5%) TCMR. In the ABM category, ABMR was the most common (10.4%) when combining hyperacute, acute, and chronic. In addition, 167 cases (6.3%) were diagnosed in MVI, DSA negative, and C4d negative, and transplant glomerulopathy accounted for 115 cases (4.3%). Mixed category consisted of 433 cases (16.2%), with MVI and TCMR (133 cases, 5.0%) being the most common, followed by MVI and borderline change (123 cases, 4.6%), ABMR

and TCMR (94 cases, 3.5%), and ABMR and borderline change (83 cases, 3.1%).

Pathologic diagnostic entities of drug toxicity in transplant biopsy

Drug toxicity was diagnosed in 944 cases (13.4%) among 337 recipients, with a 2.3 times higher incidence in males. The diagnoses were calcineurin inhibitor nephrotoxicity (267 cases, 79.2%), cyclosporine A nephrotoxicity (62 cases, 18.4%), FK506 nephrotoxicity (4 cases, 1.2%), and immunosuppressant nephrotoxicity (4 cases, 1.2%).

Pathologic diagnostic entities of glomerular disease in transplant biopsy

Glomerular disease was classified according to the pathologic diagnostic entity, with 1,164 cases diagnosed in 707 recipients (Table 4). IgA-related GN (526 cases, 45.2%) accounted for the highest frequency. This was followed by podocytopathy (146 cases, 12.5%), mesangial change/GN (140 cases, 12.0%), and diabetic renal disease (112 cases, 9.6%). Further details on each glomerular diagnostic entity are provided in [Supplementary Table S1](#).

Classification of glomerular diseases by morphologic category

Upon classification of glomerular diseases by morphologic category, proliferative GN (680 cases, 58.4%) was the most common, followed by non-proliferative (413 cases, 35.5%), sclerosing (68 cases, 5.8%), and mixed GN (3 cases, 0.3%). Among proliferative GN types, IgA nephropathy (IgAN) was the most common (523 cases, 76.9%) (Table 5). Among non-proliferative GN types, DMN was the most common (109 cases, 26.4%), followed by focal segmental glomerulosclerosis (FSGS) (75 cases, 18.2%), and membranous GN (MGN) (55 cases, 13.3%) (Table 5). The scler-

Table 4. Classification of 1,164 transplant biopsies according to diagnostic categories of glomerular disease

Glomerular disease	No. of patients (%)	Major and minor diagnosis, n (%)
IgA-related GN	310 (43.8)	526 (45.2)
Podocytopathy	102 (14.4)	146 (12.5)
Mesangial changes/GN	71 (10.0)	140 (12.0)
Diabetic renal disease	50 (7.1)	112 (9.6)
End stage GN	47 (6.6)	68 (5.8)
Membranous GN	39 (5.5)	55 (4.7)
Membranoproliferative GN	33 (4.7)	48 (4.1)
Other glomerular change	23 (3.3)	30 (2.6)
Crescentic GN	10 (1.4)	12 (1.0)
SLE, autoimmune disease	8 (1.1)	10 (0.9)
Endocapillary proliferative GN	6 (0.8)	7 (0.6)
Hereditary disease of GBM	3 (0.4)	5 (0.4)
Amyloidosis	2 (0.3)	2 (0.2)
Fibrillary GN	1 (0.1)	1 (0.1)
Monoclonal Ig deposition disease	1 (0.1)	1 (0.1)
Mixed GN	1 (0.1)	1 (0.1)
Total number	707 (100)	1,164 (100)

GN, glomerulonephritis; SLE, systemic lupus erythematosus; GBM, glomerular basement membrane.

rosing GN category included chronic sclerosing GN (41 cases, 60.3%) and diffuse glomerulosclerosis (27 cases, 39.7%). In the mixed GN category, immunotactoid GN, light chain deposition disease, and mixed IgA and MGN were each diagnosed in one case (33.3%).

Classification of glomerular diseases by etiologic category

Classification of glomerular diseases by etiologic category showed the following distribution: primary (941 cases, 80.8%), secondary (142 cases, 12.2%), unknown (41 cases, 3.5%), mixed (35 cases, 3.0%), and hereditary (5 cases, 0.4%). For primary GN, IgAN (523 cases, 55.6%) accounted for the highest, followed by FSGS (75 cases, 8.0%) and focal glomerulosclerosis (66 cases, 7.0%) (Table 6). In secondary GN, DMN (109 cases, 76.8%) was the most common, followed by lupus nephritis (10 cases, 7.0%) (Table 6). Among mixed GN, diffuse glomerulosclerosis (27 cases, 77.1%) was predominant. Hereditary GN included type IV collagen disease (4 cases, 80.0%) and focal and segmental thinning of glomerular basement membrane (1 case, 20.0%).

Pathologic diagnostic entities of tubulointerstitial disease in transplant biopsy

In transplant biopsies, 1,812 cases (25.7%) were diagnosed as tubulointerstitial diseases in 701 recipients (Table 7). The most prevalent was interstitial fibrosis/tubular atrophy (IF/TA) (971 cases, 53.6%), followed by acute tubular necrosis (483 cases,

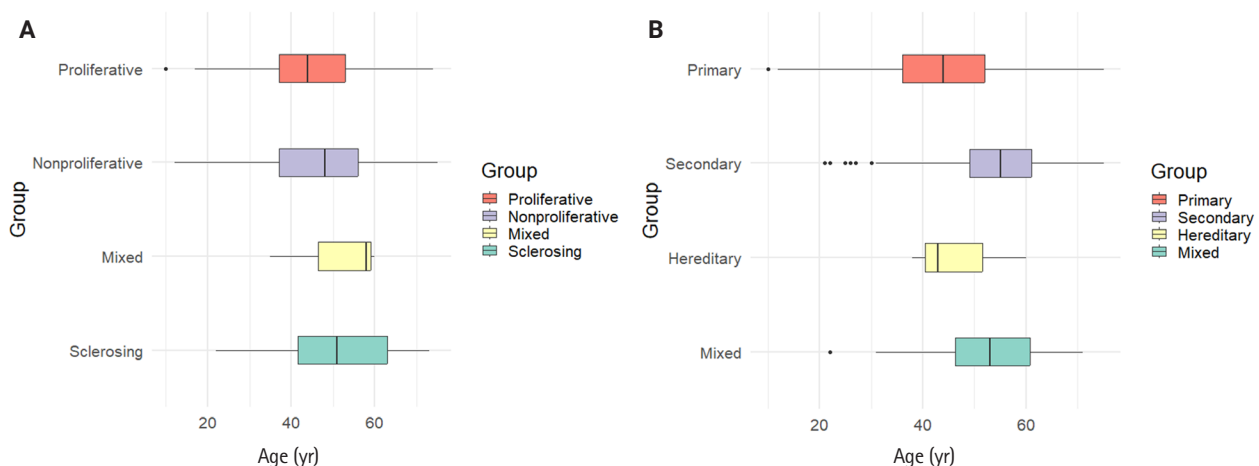


Fig. 6. Box plot analysis of age distribution according to glomerular disease categories in transplant kidney biopsy. (A) Morphologic categories of glomerular disease. The mean age and standard deviation of each group was as follows: proliferative (44.3 ± 11.2 years), non-proliferative (46.2 ± 13.9 years), mixed (51 ± 11.3 years), and sclerosing (51.4 ± 12.8 years) ($p = .001$). (B) Etiologic categories of glomerular disease. The mean age and standard deviation of each group was as follows: primary (43.9 ± 11.9 years), secondary (53.5 ± 12.4 years), hereditary (47 ± 9.42 years), and mixed (52.3 ± 12.8 years) ($p < .001$).

Table 5. Classification of transplant biopsies according to diagnostic categories of proliferative (n = 680) and non-proliferative (n = 413) glomerular diseases

	No. of cases (%)
Proliferative glomerular disease	
IgA nephropathy	523 (76.9)
Mesangial hypercellularity	50 (7.4)
Membranoproliferative GN	42 (6.2)
Mesangial proliferative GN	24 (3.5)
Lupus nephritis	10 (1.5)
Focal proliferative GN	6 (0.9)
Postinfectious GN	6 (0.9)
Diffuse proliferative lobular GN	5 (0.7)
Pauci-immune crescentic GN	4 (0.6)
Henoch-Schönlein nephritis	3 (0.4)
C1q nephropathy	2 (0.3)
ANCA-associated GN	2 (0.3)
Endocapillary proliferative GN	1 (0.1)
Focal glomerular crescent formation	1 (0.1)
Immune-complex mediated GN	1 (0.1)
Total	680 (100)
Non-proliferative glomerular disease	
Diabetic nephropathy	109 (26.4)
FSGS	75 (18.2)
Focal glomerulosclerosis	66 (16.0)
Mesangial Ig deposition	58 (14.0)
Membranous GN	55 (13.3)
Minor glomerular change	29 (7.0)
Necrotizing GN	5 (1.2)
Type IV collagen disease	4 (1.0)
Minimal change disease	3 (0.7)
Linear IgG staining in the GBM/TBM	2 (0.5)
Amyloidosis	2 (0.5)
IgM nephropathy	1 (0.2)
Degenerative changes of epithelial/endothelial cells	1 (0.2)
Focal and segmental thinning of GBM	1 (0.2)
Idiopathic nodular glomerulosclerosis	1 (0.2)
Ischemic nephropathy	1 (0.2)
Total	413 (100)

GN, glomerulonephritis; ANCA, anti-neutrophil cytoplasmic antibody; FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; TBM, tubular basement membrane.

26.7%) and virus infection (139 cases, 7.7%). More detailed information on tubulointerstitial diagnostic entities is available in [Supplementary Table S2](#).

Table 6. Classification of transplant biopsies according to diagnostic categories of primary (n = 941) and secondary (n = 142) glomerular diseases

	No. of cases (%)
Primary glomerular disease	
IgA nephropathy	523 (55.6)
FSGS	75 (8.0)
Focal glomerulosclerosis	66 (7.0)
Mesangial Ig deposition	58 (6.2)
Membranous GN	55 (5.8)
Mesangial hypercellularity	50 (5.3)
Membranoproliferative GN	42 (4.5)
Minor glomerular change	29 (3.1)
Mesangial proliferative GN	24 (2.6)
Focal proliferative GN	6 (0.6)
Pauci-immune crescentic GN	4 (0.4)
Minimal change disease	3 (0.3)
C1q nephropathy	2 (0.2)
IgM nephropathy	1 (0.1)
Degenerative changes of epithelial/endothelial cells	1 (0.1)
Idiopathic nodular glomerulosclerosis	1 (0.1)
Immune-complex mediated GN	1 (0.1)
Total number	941 (100)
Secondary glomerular disease	
Diabetic nephropathy	109 (76.8)
Lupus nephritis	10 (7.0)
Postinfectious GN	6 (4.2)
Necrotizing GN	5 (3.5)
Henoch-Schönlein nephritis	3 (2.1)
Amyloidosis	2 (1.4)
Linear IgG staining in the GBM/TBM	2 (1.4)
ANCA-associated crescentic GN	2 (1.4)
Light chain deposition disease	1 (0.7)
Ischemic nephropathy	1 (0.7)
Immunotactoid GN	1 (0.7)
Total number	142 (100)

FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; GBM, glomerular basement membrane; TBM, tubular basement membrane; ANCA, anti-neutrophil cytoplasmic antibody.

Pathologic diagnostic entities of vascular disease in transplant biopsy

The number of vascular diseases was 278 cases (3.9%) diagnosed among 76 recipients ([Table 7](#)). Arteriosclerosis was the most commonly diagnosed entity (145 cases, 52.2%), followed by arterial hyalinosis (60 cases, 21.6%), arterionephrosclerosis (47 cases, 16.9%), thrombotic microangiopathy (TMA) (18 cases,

6.5%), and isolated vascular lesion (8 cases, 2.9%). More detailed information on each vascular diagnostic entity can be found in [Supplementary Table S3](#).

Renal disease in transplant nephrectomy

A total of 176 diagnoses were made in 116 recipients who underwent transplant nephrectomy ([Table 8](#)). The mean age was 46.1 ± 11.9 years and the sex ratio was 1.42:1. When all diseases were categorized according to disease types, "Others" (60 cases, 34.1%) was the most common, followed by rejection (59 cases, 33.5%), tubulointerstitial (25 cases, 14.2%), vascular (17 cases, 9.7%), glomerular (10 cases, 5.7%), and drug toxicity (5 cases,

2.8%). The age and sex distribution of all recipients who underwent nephrectomy is shown in [Fig. 2B](#). The number of recipients increased significantly in the 30s, passed through the 40s, peaked in the 50s, and then decreased after the 60s.

Trends of renal disease in transplant nephrectomy

Decadal changes of nephrectomies performed for renal disease

[Fig. 7](#) illustrates the changes in renal disease types over a 30-year period. The number of nephrectomies remained stable across the three decades: 1990s (30 recipients), 2000s (38 recipients), and 2010s (32 recipients).

Decadal changes of nephrectomies classified by renal disease types

When analyzing the change in disease frequency over the 30 years, rejection in nephrectomy steadily decreased from the 1990s (20 cases, 66.7%) to the 2000s (18 cases, 47.4%) and the 2010s (5 cases, 15.6%) ([Fig. 7](#)). There were no significant changes in tubulointerstitial and vascular diseases over time. "Others" increased from the 1990s (4 cases, 13.3%) to the 2000s (10 cases, 26.3%) and the 2010s (18 cases, 56.3%).

Differences in renal disease types by age group

The distribution of renal diseases in nephrectomy specimens was analyzed by age groups ([Fig. 8](#)). All recipients younger than 20 years were diagnosed with rejection. Characteristically, the proportion of "Others" diagnosed after the 20s was 28.6% and increased with age, reaching the highest frequency (66.7%) in the ≥ 60 years group ($p = .011$). When the categories of rejection were analyzed by age ([Fig. 9](#)), TCM (40.2 ± 12.1 years) and ABM (42.9 ± 14.9 years) were found in younger recipients, while

Table 7. Classification of transplant biopsies according to diagnostic categories of tubulointerstitial (n = 1,812) and vascular (n = 278) diseases

	No. of cases (%)
Tubulointerstitial disease	
Interstitial fibrosis/tubular atrophy	971 (53.6)
Acute tubular necrosis	483 (26.7)
Virus infection	139 (7.7)
Pyelonephritis	118 (6.5)
Other	65 (3.6)
Tubulointerstitial nephritis	35 (1.9)
Ig deposition disease	1 (0.1)
Total number	1812 (100)
Vascular disease	
Arteriosclerosis	145 (52.2)
Arterial hyalinosis	60 (21.6)
Arterionephrosclerosis	47 (16.9)
Thrombotic microangiopathy	18 (6.5)
Isolated vascular lesion	8 (2.9)
Total number	278 (100)

Table 8. Demographic data of 116 patients who underwent transplant nephrectomy

Category	All diseases	Rejection	Drug toxicity	Glomerular disease	Tubulointerstitial disease	Vascular disease	Others	p-value (excluding others)	p-value (including others)
No. of patients	116 (100)	46 (39.7)	0	6 (5.2)	17 (14.7)	3 (2.6)	44 (37.9)	-	-
Major and minor diagnoses	176 (100)	59 (33.5)	5 (2.8)	10 (5.7)	25 (14.2)	17 (9.7)	60 (34.1)	-	-
Age (yr)	46.1 ± 11.9	41.9 ± 13.3	-	47.7 ± 9.1	47.5 ± 8.4	50.3 ± 4.0	49.4 ± 11.2	.239	.041
Male:female ratio	1.42:1	1.3:1	-	2:1	1.43:1	0.5:1	1.59:1	.815	.883

Values are presented as number (%) or mean $\pm$ standard deviation.

mixed category (53.5 ± 9.8 years) was found in older recipients.

Pathologic diagnostic entities of renal disease in transplant nephrectomy

The most common diagnosis in patients who underwent nephrectomy was rejection (59 cases, 33.5%), of which chronic TCMR (24 cases, 40.7%) was the most common, followed by acute TCMR. Renal infarction was the second most common diagnosis, occurring in 35 cases (19.9%). Drug toxicity was

diagnosed in five cases of calcineurin inhibitor nephrotoxicity. Among glomerular diseases (10 cases, 5.7%), chronic sclerosing GN (3 cases, 30.0%) was the most common. When glomerular diseases were classified by morphologic category, the distributions were as follows: proliferative (3 cases, 30%), non-proliferative (2 cases, 20%), and sclerosing GN (5 cases, 50%). When classified by etiologic category, the distribution of each category was as follows: primary (3 cases, 30%), unknown (3 cases, 30%), secondary (2 cases, 20%), and mixed GN (2 cases, 20%). Among

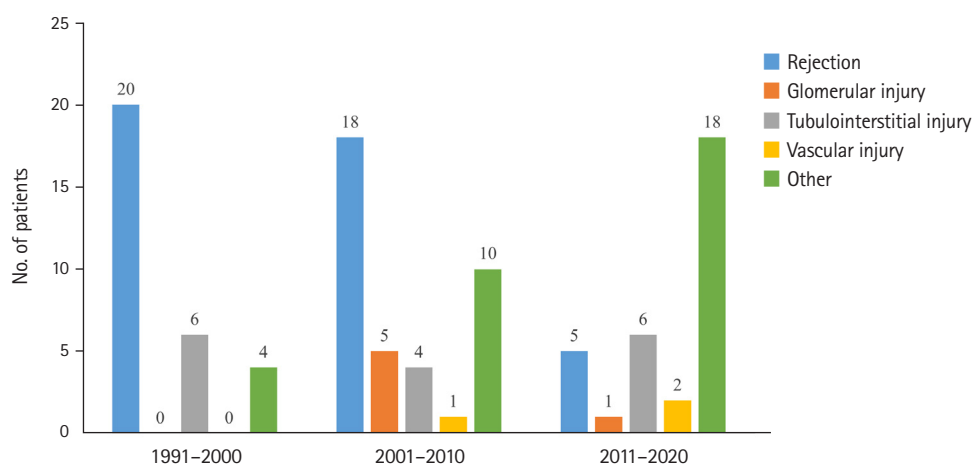


Fig. 7. Three-decade trends in renal disease types in 100 patients undergoing transplant nephrectomy. They were analyzed for three consecutive decades (1991–2000, 2001–2010, and 2011–2020).

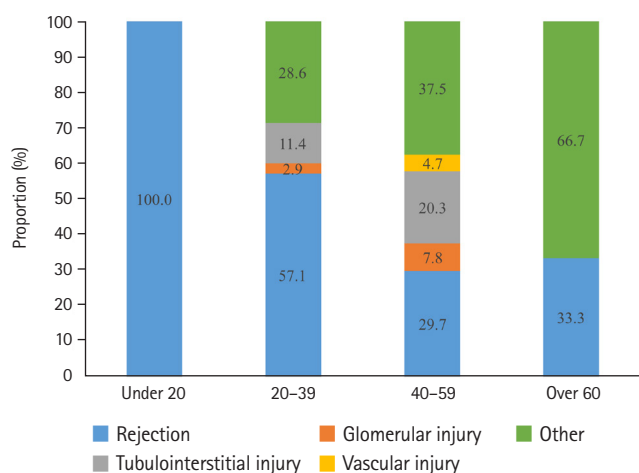


Fig. 8. Proportional analysis of renal disease types among four age groups in transplant nephrectomies. Patients were stratified into four groups; under 20 years, 20–39 years, 40–59 years, and over 60 years. The proportion of other diseases diagnosed after the age of 20 increased with age, reaching the highest frequency in the over 60 age group ($p = .011$).

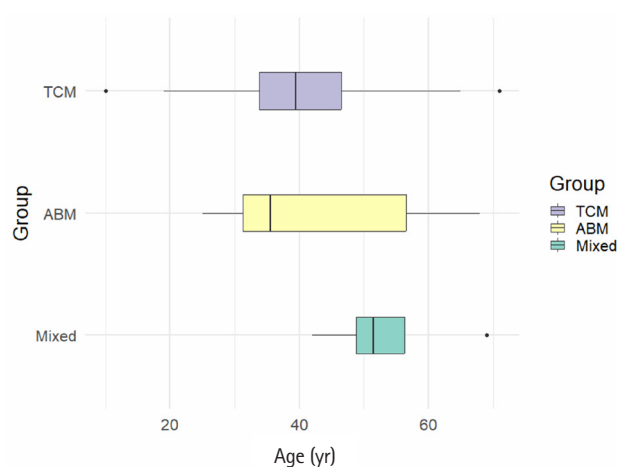


Fig. 9. Box plot analysis of age distribution according to rejection categories in transplant nephrectomy. The mean age and standard deviation of each group was as follows: T-cell mediated (TCM) (40.2 ± 12.1 years), antibody mediated (ABM) (42.9 ± 14.9 years), and mixed (53.5 ± 9.8 years) ($p = .187$).

the tubulointerstitial diseases (25 cases, 14.2%), IF/TA (12 cases, 48%) was the most common, followed by pyelonephritis (7 cases, 28%). The diagnoses of vascular diseases (17 cases, 9.7%) included arteriosclerosis (12 cases, 70.6%) and TMA (5 cases, 29.4%). More detailed information on each pathologic diagnostic entity is available in [Supplementary Table S4](#).

DISCUSSION

In this study, we retrospectively analyzed all renal diseases diagnosed through pathological examination in 7,229 transplanted kidneys from 4,188 recipients over the past 33 years. Among them, rejection was a major threat to transplanted kidneys both in the biopsy (2,664 cases, 37.8%) and the nephrectomy (59 cases, 33.5%), and these findings are similar to those in a previous report [17,19–22,26]. Our study also indicated a predominance of male recipients across all disease types with the exception vascular disease in nephrectomies. Previous reports have also reported a male predominance in renal disease after transplantation [10,21,27–29].

In our biopsy group, rejection accounted for the highest proportion in all age groups, with the highest frequency of 52.6% in recipients aged 40–59 years. Furthermore, we found that TCM category (42.0±11.6 years) was predominantly observed in younger recipients compared to ABM (47.5±11.0 years) and mixed (48.1±11.7 years) ($p < .001$) categories. Previous studies have shown that recipients with mixed rejection (mean age, 41.9 years) were younger than those with TCMR (47.4) or ABMR (49.7) ($p = .012$), and exhibited the worst prognosis [21,30]. Among the rejection cases in our biopsies, TCM category was predominant ($n = 1,602$, 60.1%), with borderline change, indicating suspicion of acute TCMR, accounting for 34.3%, more frequently diagnosed than acute (21.3%) or chronic TCMR (4.5%). This contrasts with previous reports, which found borderline changes in biopsies from functioning allografts to be diagnosed in 9% to 13.8% of cases [19,27]. This discrepancy may be due to the fact that the protocol biopsy was performed routinely on day 14 after transplantation in this study.

Among our biopsies, TCMR (25.8%) was diagnosed more commonly than ABMR (10.4%) and mixed TCMR/ABMR (3.5%). Although the results from various studies on pathological diagnosis in transplant recipients were diverse, and rejection was the most common diagnosis [21,22,28]. A 4-year analysis of allograft biopsy reports from 2017 in Türkiye [28] revealed that acute TCMR (14.1%) was diagnosed more commonly than

acute ABMR (4%) and mixed rejection (5.7%). However, data from 139 transplant recipients reported in Korea from 2006 to 2018, ABMR (41.7%) was more common than TCMR (34.5%) or mixed rejection (23.7%), where mixed rejection had the worst prognosis [21]. According to the study by Halloran et al. [22], TCMR frequently occurred in the early stages and diminished over time, and was absent after 10 years, which was hypothesized as adaptive T-cell tolerance due to long-term immunosuppression. Conversely, ABMR became common after the first month and increased over time, and was strongly associated with graft failure unlike TCMR [22]. Such discrepancies may be due to the fact that our study including all diagnosed cases regardless of graft function, whereas most other studies focused on cases with graft failure.

Many studies have documented the various causes of renal graft failure after KT [13–20], revealing considerable regional and temporal variations. Rejection was the predominant cause of allograft loss from the 1970s to the 1990s [16,18], followed by vascular thrombosis, rejection, GN, IF/TA, and polyoma virus nephropathy from the 1990s to the 2010s [13,15,18,19]. Betjes et al. [20] reported that rejection was the primary cause of graft failure, and found that TCMR was the most common in the first 5 years after transplantation, but its incidence decreased thereafter, whereas ABMR incidence increased annually by 1.1%. In a multicenter study in Australia during the 2010s, rejection was the cause of graft loss in 47.5%, with chronic ABMR (37.4%) being the most common [17]. Also, a French report on 4,783 transplant recipients in 2018 identified ABMR as the most common (31.69%) cause of graft loss [14].

The incidence of drug toxicity in our study increased with age, peaking in those over 60 years (11.9%) compared to other age groups ($p = .047$). Similar findings were reported in other studies, indicating that drug-induced nephrotoxicity was more frequent in recipients over 60 years with more comorbidities [31], though it was less frequent as a cause of renal allograft loss [13,14,16,17]. Calcineurin inhibitor nephrotoxicity occurred in 76.4% at 1 year and 93.5% at 5 years after transplantation [32].

The glomerular (25.0%) and tubulointerstitial diseases (22.9%) in our biopsies, constituted the highest proportions in recipients under 20 years compared to other age groups ($p < .001$). Compared with our native kidney data from the same period, glomerular disease was also most common in those under 20 years, whereas tubulointerstitial disease was most common in those over 60 years of age [33]. However, the incidence of tubulointerstitial diseases in transplant biopsy did not vary significantly

across age groups (range, 16.5% to 22.9%). Although no studies were identified regarding tubulointerstitial disease in renal allografts in existing literature, our comparison with native kidney data revealed a higher frequency of tubulointerstitial disease in younger recipients of allograft kidneys.

Recurrent or de novo glomerular disease is an important cause of graft dysfunction and subsequent loss. When we categorized GN according to morphologic and etiologic categories in this study, proliferative (58.2%) and primary GN (80.8%) were the most common in renal graft biopsies, similar to findings from our native kidney data (proliferative [57.9%] and primary GN [72.8%], respectively) [33]. In terms of morphologic categories, recipients with proliferative or non-proliferative GN were younger (early 40s) than recipients with mixed or sclerosing GN (late 40s) in both our transplant and native kidney studies [33]. In proliferative GN, IgAN was the most common (523 cases, 76.9%). In non-proliferative GN, DMN was the most common (109 cases, 26.4%), followed by FSGS (75 cases, 18.2%), and focal glomerulosclerosis (66 cases, 16.0%). Previous studies have also shown that IgAN and FSGS are major contributors to graft failure, either recurrent or de novo [10,12,13,34–36]. In renal transplant recipients with recurrent renal disease, FSGS (26%) was reported to be the most common, followed by DMN (22%) [37]. El-Zoghby et al. [13] reported GN as the leading cause of renal graft loss (36.6%). An international, multicenter collaboration study of TANGO (Post-Transplant Glomerular Disease) reported that overall recurrence of FSGS was 32%, with a median recurrence time of 1.5 months, and 39% of them lost their graft over a median of 5 years [34]. The TANGO study also showed that the cumulative recurrence of IgAN was 19% at 10 years and 23% at 15 years, resulting in a 3.7-fold higher risk of graft loss [35]. When classified by etiology categories, recipients with primary GN were significantly younger at diagnosis compared to others in transplant kidneys ($p < .001$). This contrasts with our native kidney data, where the mean age of those with primary GN was not significantly different from that of secondary or hereditary GN [33].

The proportion of tubulointerstitial disease in this transplant biopsy was 17.2%, with IF/TA being the most common pathological finding. IF/TA, previously termed chronic graft nephropathy, is reported to be prevalent in approximately 40%–65% of kidney transplant recipients [38]. Chronic graft nephropathy typically occurred a median of three months after transplantation and was present in 94.2% of recipients by one year due to immunologic and ischemic results [32]. In our study, the total

number of vascular disease was 278 cases (3.9%), with arteriosclerosis accounting for the highest proportion. Although vascular disease in transplant recipients is less explored than other disease types, vascular thrombosis has been identified as a main cause for early graft failure [15,18].

In our nephrectomy kidneys, the number did not change significantly over the three decades: 1990s (30 recipients), 2000s (38 recipients), and 2010s (32 recipients)). When analyzing the change in disease frequency over the 30 years, “Others” increased from the 1990s (4 cases, 13.3%) to the 2000s (10 cases, 26.3%) and the 2010s (18 cases, 56.3%). The most common diagnosis in “Others” was renal infarction (35 cases, 58.3%). Previous studies have also reported infarction and vascular complications as major indications for transplant nephrectomy with varying incidences (8.2% [39], 10.7% [29], 15% [40]), but rejection was generally identified as the main cause [28,29,39].

The rejection in our nephrectomy decreased progressively from the 1990s (20 cases, 66.7%) to the 2000s (18 cases, 47.4%) and the 2010s (5 cases, 15.6%). Rejection was observed in 59 cases (33.5%), with chronic TCMR (24 cases, 40.7%) being the most frequent diagnosis, followed by acute TCMR (16 cases, 27.1%). Our results differ from existing reports. In an analysis of 195 transplant nephrectomy cases conducted from 2005 to 2020 [39], the principal cause was graft intolerance syndrome (26.7%), followed by acute rejection (18.5%), acute infection (15.4%), and other reasons (13.3%). In the study by Bunthof et al. [29], chronic rejection (without differentiating between TCMR and ABMR) was 49% and acute rejection was 9%.

IgAN was the most common glomerular disease in our nephrectomy cases, consistent with the findings from our native nephrectomy study [33]. A study conducted in Hong Kong from 1980 to 2004 found that IgAN was common in allograft nephrectomy cases [36]. There were no significant changes in tubulointerstitial and vascular diseases over time in our analysis. There are few studies about tubulointerstitial and vascular disease in transplant nephrectomy. One study identified renal artery thrombosis as a primary cause of early transplant nephrectomy, and chronic allograft nephropathy as a cause later in the course [40]. According to Mang et al. [39], vascular complications accounted for 8.2% of nephrectomy indications. Bunthof et al. [29] reported that hypertension and thrombosis accounted for 7.1% of graft failure causes, while nephrolithiasis accounted for 1.0%.

Limitations of this study include: First, the lack of clinical data on recipients prevented sufficient consideration of clinical

factors when analyzing transplant diagnoses. Second, as it was a single-center analysis, the results may have limited and might not accurately reflect the real facts. Third, the small number of nephrectomy cases may limit the statistical validity of the results when divided into several groups for analysis. Finally, although the updated Banff classification for transplant rejection has been applied for diagnosis since 1997, a complete review and reevaluation of previous diagnoses has not been performed, which may limit its diagnostic impact.

This comprehensive analysis of allograft kidney pathology over 33 years provides valuable insights into the post-transplant renal diseases in South Korea. Our study also provides important data for understanding the pathologies diagnosed in transplanted kidneys, especially rejection and various diseases. However, it is a single-center study, so multicenter collaboration is needed to obtain more comprehensive results.

In conclusion, this study provides valuable insights into renal disease after KT in South Korea. The number of transplant biopsies increased over 33 years, but nephrectomies remained unchanged. Rejection was most common in all age groups in biopsies, but it decreased with age in nephrectomy cases. Among the rejections, the TCM category was the most common and was observed in younger recipients than the other types.

Supplementary Information

The Data Supplement is available with this article at <https://doi.org/10.4132/jptm.2026.03.28>.

Ethics Statement

This study was approved by the Institutional Review Board (IRB) of The Catholic University of Korea, Seoul St. Mary's Hospital (IRB approval no. 2024-3117-0001, date of approval: 10 January 2025). It was performed in accordance with the Declaration of Helsinki. The requirement for written informed consent was waived due to retrospective nature of the study.

Availability of Data and Material

The data presented in this study are available from the corresponding author upon 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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Increased Toll-like receptor 9 expression associated with cellular proliferation and poor prognosis in pleural mesothelioma

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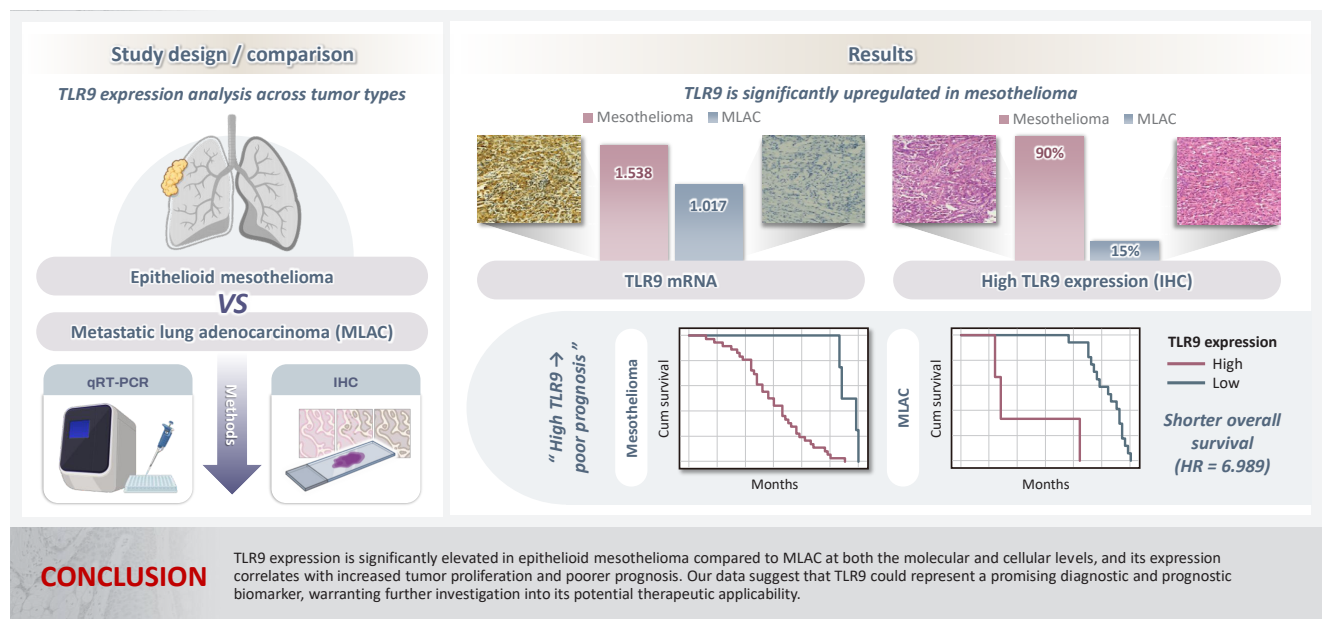
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Graphical abstract



Increased Toll-like receptor 9 expression associated with cellular proliferation and poor prognosis in pleural mesothelioma

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Background: Pleural mesothelioma is an aggressive malignancy with a poor prognosis. The epithelioid subtype is the most common and can be challenging to distinguish from metastatic lung adenocarcinoma (MLAC). The role of Toll-like receptor 9 (TLR9) in the progression of pleural mesothelioma remains unclear. **Methods:** A total of 30 pleural biopsy specimens were collected, comprising 10 cases of pleural epithelioid mesothelioma and 20 cases of MLAC. The mRNA expression levels of TLR9 and proliferating cell nuclear antigen (PCNA) were quantified. In addition, sixty archived formalin-fixed, paraffin-embedded tissue blocks (40 epithelioid mesothelioma and 20 MLAC) were analyzed via immunohistochemistry using an anti-TLR9 antibody in relation to various clinicopathological parameters. **Results:** TLR9 expression was significantly higher in epithelioid mesothelioma cases than in MLAC cases ($p < .001$), with mean values of 1.54 ± 0.09 and 1.02 ± 0.08 , respectively. A significant positive correlation was observed between TLR9 and PCNA expression levels specifically in the epithelioid mesothelioma cohort ($p < .001$, $r = 0.8$). Furthermore, immunohistochemical analysis confirmed that high TLR9 immunoreactivity was significantly more prevalent in epithelioid mesothelioma (36/40 cases; 90%) than in MLAC (3/20 cases; 15%) ($p < .001$). Notably, elevated TLR9 expression was associated with a significantly shorter overall survival ($p = .001$). **Conclusions:** In conclusion, TLR9 expression is significantly elevated in epithelioid mesothelioma compared to MLAC at both the molecular and cellular levels, and its expression correlates with increased tumor proliferation and poorer prognosis. Our data suggest that TLR9 could represent a promising diagnostic and prognostic biomarker, warranting further investigation into its potential therapeutic applicability.

Keywords: Toll-like receptor 9; Mesothelioma, malignant; Adenocarcinoma of lung; Diagnosis, differential; Immunohistochemistry

INTRODUCTION

Pleural mesothelioma is a rare and aggressive malignancy with a poor prognosis, typically characterized by an average overall survival (OS) of 9 to 18 months [1]. The epithelioid subtype is the most prevalent and is often challenging to distinguish from metastatic lung adenocarcinoma (MLAC). The diagnostic confirmation of epithelioid mesothelioma typically relies on immu-

nohistochemical profiling using a combination of two positive mesothelial markers (e.g., calretinin, Wilms' tumor-1, or cytokeratin 5/6) and two negative markers (e.g., carcinoembryonic antigen, Ber-EP4, or MOC-31) to exclude carcinoma [2]. The considerable histological diversity of mesotheliomas, coupled with the pleura being a common site for metastatic disease, renders diagnosis based on morphology alone particularly challenging. Current treatment modalities are primarily limited to

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surgery, chemotherapy, and radiotherapy. Consequently, there is an urgent, unmet need to develop novel, effective, and tailored therapeutic strategies for pleural epithelioid mesothelioma. Although Toll-like receptor 9 (TLR9) has ushered in a new era for mesothelioma management as a form of immunotherapy, its precise role remains incompletely understood [3]. TLR9 agonists show significant potential in combination therapies, where they synergize with other agents to augment anti-tumor immune responses [4]. Despite this promise, clinical trials of these agonists have yet to meet expectations [5]. Therefore, elucidating the expression pattern of TLR9 in mesothelioma and deciphering its role in tumorigenesis—particularly in proliferation—is essential. In this study, we compare TLR9 expression in pleural epithelioid mesothelioma and MLAC and correlate this expression with patients' clinicopathological parameters.

MATERIALS AND METHODS

Sample collection

Fresh samples

Pleuroscopy was performed under local anesthesia with intravenous conscious sedation using a Karl Storz rigid thoracoscope (Tuttlingen, Germany). The entry site, typically at the 5th or 6th intercostal space, was optimized under ultrasound guidance. Approximately six biopsies were collected from visible pleural lesions. From each patient, two sample sets were obtained: one was snap-frozen at -80°C for molecular analysis, and the other was fixed in neutral buffered formalin 10% for histopathological examination.

Paraffin blocks

Sixty archival formalin-fixed paraffin-embedded (FFPE) tissue blocks were randomly selected from previously diagnosed cases at the pathology laboratories of Minia University Hospitals and Minia Oncology Centre. All specimens were obtained via thoracoscopic biopsy. The cohort consisted of 40 cases of epithelioid mesothelioma and 20 cases of MLAC.

Histopathological confirmation and immunohistochemical validation

Histopathological examination of hematoxylin and eosin-stained sections was performed to confirm the initial diagnosis and to assess key tumor characteristics, including tumor grade, the presence of tumor-infiltrating lymphocytes (TILs), and necrosis. Immunohistochemical analysis confirmed the differ-

ential expression of the studied markers, supporting the mRNA expression results.

Pathological assessment

The pathological T stage was determined for all cases according to the 8th edition of the tumor-node-metastasis (TNM) classification system [6]. Tumor grading was assessed following the 2015 World Health Organization classification of tumors of the lung and pleura [7]. The presence or absence of tumor necrosis and significant TILs was systematically recorded.

Clinicopathological data collection

Relevant clinicopathological data were extracted through a comprehensive review of medical records. Systematically collected demographic data included patient age at diagnosis and sex. Age groups were subsequently categorized based on the median age of the enrolled cases. The clinicopathological data of the patients are summarized in Table 1.

Quantitative real-time polymerase chain reaction

The relative expression levels of TLR9 and proliferating cell nuclear antigen (PCNA) mRNA were quantified using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from pleural tissue samples using RiboZol solution (AMSBIO, Cambridge, UK). RNA concentration and purity were assessed spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA); samples with a 260/280 ratio greater than 2.0 and a 260/230 ratio greater than 1.8 were considered intact and used for subsequent analysis. cDNA was synthesized from the extracted RNA, and real-time polymerase chain reaction (PCR) was subsequently performed using a commercial SYBR Green PCR kit (Qiagen, Hilden, Germany). The SYBR Green PCR kit included the provided Universal PCR Master Mix. The thermal protocol was run on DTLite Real-Time PCR System (DNA Technology, Moscow, Russia), and performed as follows: 45°C for 10 minutes, 95°C for 2 minutes, followed by 40 cycles at 95°C for 5 seconds, 60°C for 10 seconds, and 72°C for 5 seconds. Fluorescence was measured during the 72°C step for each cycle. The relative quantitation of both TLR9 and PCNA expression to glyceraldehyde 3-phosphate dehydrogenase (reference gene) were calculated using cycle threshold (Ct) method. Ct was calculated by DT master software: $\Delta\text{Ct} = \text{Ct}(\text{target gene}) - \text{Ct}(\text{reference gene})$. The $\Delta\Delta\text{Ct}$ values were calculated through the following equation = $\text{Mean } \Delta\text{Ct}(\text{epithelioid mesothelioma}) - \text{Mean } \Delta\text{Ct}(\text{MLAC})$.

Table 1. The clinicopathological data of epithelioid mesothelioma and MLAC patients (n=60)

Clinicopathological variable	Diagnosis	
	Epithelioid mesothelioma (n = 40)	MLAC (n = 20)
Age (yr)		
≤ 52	24 (77.4)	7 (22.6)
> 52	16 (55.2)	13 (44.8)
Sex		
Male	27 (73.0)	10 (27.0)
Female	13 (56.5)	10 (43.5)
Tumor grade		
Low/intermediate	11 (55.0)	9 (45.0)
High	29 (72.5)	11 (27.5)
T category of primary tumor		
T1	2 (18.2)	9 (81.8)
T2	6 (50.0)	6 (50.0)
T3	23 (88.5)	3 (11.5)
T4	9 (81.8)	2 (18.2)
TILs		
Present	19 (59.4)	13 (40.6)
Absent	21 (75.0)	7 (25.0)
Tumor necrosis		
Present	18 (69.2)	8 (30.8)
Absent	22 (64.7)	12 (35.3)
Overall survival		
Alive	8 (32.0)	17 (68.0)
Dead	32 (91.4)	3 (8.6)

Values are presented as number (%).

All MLAC cases with pleural involvement are classified as stage IV (M1a) disease. The T category refers to the size and extent of the primary lung tumor in the lung.

MLAC, metastatic lung adenocarcinoma; TIL, tumor-infiltrating lymphocyte.

The TLR9 real-time PCR forward primer was TTCCCTG-TAGCTGCTGTCC, reverse primer was ACAGCCAGTTG-CAGTTCACC.

The PCNA real-time PCR forward primer was CCATCCT-CAAGAAGGTGTTGG, reverse primer was GTGTCCCATATC-CGCAATTTAT.

Immunohistochemistry

Four-micrometer tissue sections were cut from the FFPE blocks and mounted on positively charged slides. Immunohistochemistry (IHC) was performed on an automated immunostainer (Ventana BenchMark GX, Ventana Medical Systems, Inc., Tucson, AZ, USA) according to the manufacturer's protocol. Anti-

gen retrieval was conducted using a Tris-based buffer (pH 7.6). Sections were incubated with a mouse anti-human monoclonal anti-TLR9 antibody [clone 26C593.2] (dilution 1:100, catalog number ab134368, Abcam, Cambridge, UK). Appropriate positive and negative controls were included in each run to ensure staining specificity and accuracy.

Evaluation of immunohistochemical staining

Immunohistochemical evaluation was performed independently by two pathologists (D.M.T. and M.F.G.). Staining was assessed based on the intensity and localization according to the method described by Gao et al. [8]. A semi-quantitative scoring system was employed, evaluating both the proportion and the staining intensity of immunoreactive cells. The proportion of stained tumor cells was scored as follows: 0 (negative), 1 (<10% stained tumor cells), 2 (10%–50% stained tumor cells), and 3 (>50% stained tumor cells). The staining intensity was scored on a scale of 0 (no staining), 1 (weak cytoplasmic staining, light yellow), 2 (moderate cytoplasmic staining, yellowish brown), and 3 (strong cytoplasmic staining, brown). A final staining index was calculated for each case using the formula: Staining index = (Intensity score) + (Proportion score).

Based on this index, cases were categorized as follows: A staining index of ≥ 4 indicated high TLR9 expression, whereas a staining index of < 4 indicated low TLR9 expression [8].

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences ver. 20.0 (IBM Corp., Armonk, NY, USA). The association between TLR9 expression and clinicopathological parameters was evaluated using the chi-square test or Fisher's exact test, as appropriate. Survival curves were generated using the Kaplan-Meier method, and differences were compared with the log-rank test. The prognostic impact of TLR9 expression was further assessed using univariate and multivariate Cox regression. Results are presented as hazard ratio (HR) with corresponding 95% confidence interval (CI). A p-value $< .05$ was deemed statistically significant.

RESULTS

Expression values of TLR9 mRNA in pleural epithelioid mesothelioma versus MLAC

qRT-PCR analysis revealed a significantly higher mRNA expression level of TLR9 in epithelioid mesothelioma cases (1.54

± 0.09) compared to MLAC cases (1.02 ± 0.08) ($p < .001$) (Supplementary Fig. S1). PCNA mRNA levels were elevated in both groups. The $\Delta\Delta Ct$ values were 0.63 for TLR9 and 0.08 for PCNA. Notably, a strong and significant positive correlation between TLR9 and PCNA expression was identified specifically within the epithelioid mesothelioma cohort ($p < .001$, $r = 0.8$) (Supplementary Fig. S2).

Immunohistochemical expression of TLR9 in pleural epithelioid mesothelioma and MLAC

Immunohistochemical analysis revealed high TLR9 expression, localized to the cytoplasm of malignant cells, in 36 of 40 (90%) epithelioid mesothelioma cases compared to only three of 20 (15%) MLAC cases (Fig. 1). In epithelioid mesothelioma,

high TLR9 expression showed a significant association with advanced T category of the primary tumor ($p = .045$) (Fig. 2). Conversely, a significant inverse relationship was observed between TLR9 expression and T category of the primary tumor in MLAC ($p = .014$). No other significant associations were found between TLR9 expression and the remaining clinicopathological parameters. A detailed summary of these associations is provided in Table 2.

Survival analysis and prognostic significance

The OS follow-up period for all 60 cases of pleural malignancy ranged from 6 to 60 months. The mean OS was 37.48 ± 1.53 months, and the median survival time was 38 months. In epithelioid mesothelioma, high TLR9 expression was signifi-

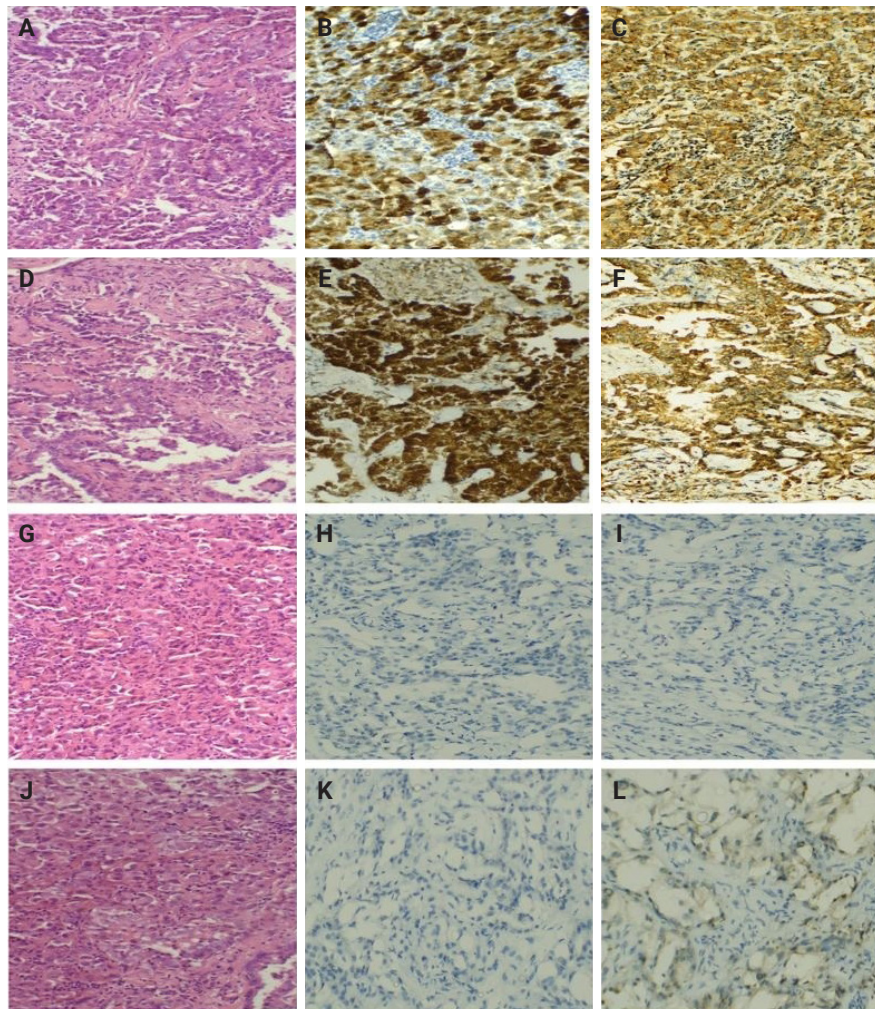


Fig. 1. (A–F) Mesothelioma: H&E staining of epithelioid mesothelioma, positive calretinin expression and high Toll-like receptor 9 (TLR9) expression, respectively. (G–L) Metastatic adenocarcinoma: H&E staining of metastatic lung adenocarcinoma, negative calretinin expression and negative/low TLR9 expression, respectively. (A, D, G, J) H&E. (B, E, H, K) Calretinin. (C, F, I, L) TLR9.

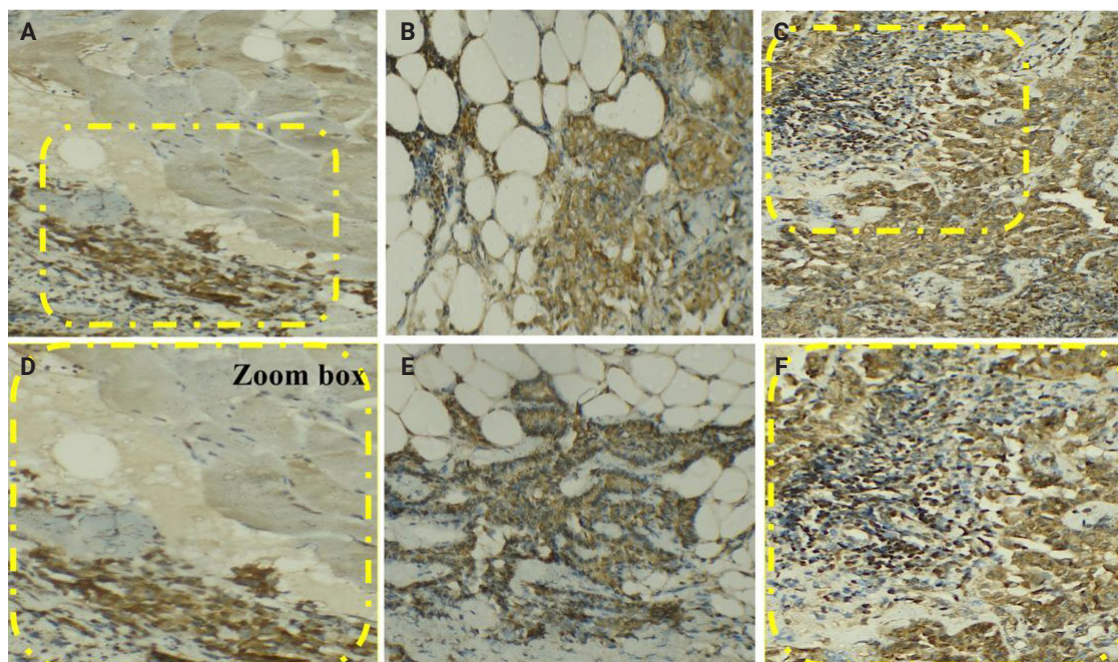


Fig. 2. Expression of Toll-like receptor 9 (TLR9) in epithelioid mesothelioma cases with tumor invasion and inflammation. High TLR9 expression in mesothelioma with muscle invasion (A, D), fat invasion (B, E), and in mesothelioma and inflammatory cells (C, F). (A) Positive TLR9 expression in mesothelioma cells invading the surrounding muscle. (B) Positive TLR9 expression in mesothelioma cells invading the surrounding fat. (C) High TLR9 expression in inflammatory cells and mesothelioma cells. (D) Yellow zoom box showing the high expression of TLR9. (E) Another case showing positive TLR9 expression in mesothelioma cells invading the surrounding fat. (F) Yellow zoom box showing the high expression of TLR9 in inflammatory cells and mesothelioma cells.

cantly associated with shortened OS (Fig. 3). Notably, 88.8% of mesothelioma patients with high TLR9 expression had died at the end of the follow-up period (log-rank test, $p = .001$). In contrast, no significant association was observed between TLR9 expression and OS in the MLAC cohort (Table 2).

Univariate and multivariable Cox regression analysis

Next, we considered clinicopathological variables (age, sex, tumor grade, tumor stage, inflammation, and tumor necrosis) in univariate Cox regression models for OS. Subsequently, a multivariable Cox regression model was constructed incorporating these variables along with TLR9 expression status. Although based on a relatively small cohort, the analysis suggested that high TLR9 expression may be independently associated with poorer prognosis in mesothelioma cases (HR, 6.989; 95% CI, 2.148 to 22.738). Given the sample size, these findings should be interpreted as exploratory and warrant further validation in larger cohorts (Table 3).

DISCUSSION

Previous studies reported that TLR3, TLR7, and TLR9 could be targetable therapy in mesothelioma [9,10]. Furthermore, the well-known TLR9 agonist, cytosine-phosphoguanine oligodeoxynucleotides induced antitumor activity against peritoneal mesothelioma in an animal model [3], but still the role of TLR9 in pleural mesothelioma is undermined.

In this study, a significantly high TLR9 expression was detected in epithelioid mesothelioma by using PCR and IHC, suggesting a potential diagnostic value. Additionally, the positive correlation found between TLR9 and PCNA mRNA expression in epithelioid mesothelioma ($p < .001$, $r = 0.8$) suggesting a possible involvement of TLR9 in tumor proliferation. Aligned with our study, high TLR9 mRNA value was previously reported by various studies to be linked with tumor proliferation in several cancers [11,12].

To note, this study represents the first investigation of the immunohistochemical expression of TLR9 in pleural mesothelioma and suggests that TLR9 may play a role in mesothelioma

Table 2. Immunohistochemical expression of TLR9 and its association with clinicopathological data

Clinicopathological variable	No.	TLR9 expression in epithelioid mesothelioma		p-value	TLR9 expression in MLAC		p-value
		Low (n = 4)	High (n = 36)		Low (n = 17)	High (n = 3)	
Age (yr)							
≤ 52	31	4 (12.9)	20 (64.5)	.136	7 (22.6)	0	.521
> 52	29	0	16 (55.2)		10 (34.5)	3 (10.3)	
Sex							
Male	37	2 (5.4)	25 (67.6)	.584	8 (21.6)	2 (5.4)	>.99
Female	23	2 (8.7)	11 (47.9)		9 (39.1)	1 (4.3)	
Tumor grade							
Low/intermediate	20	2 (10.0)	9 (45.0)	.300	9 (45.0)	0	.218
High	40	2 (5.0)	27 (67.5)		8 (20.0)	3 (7.5)	
T category of primary tumor							
T1	11	1 (9.1)	1 (9.1)	.045	9 (81.8)	0	.014
T2	12	2 (16.7)	4 (33.3)		6 (50.0)	0	
T3	26	1 (3.8)	22 (84.7)		1 (3.8)	2 (7.7)	
T4	11	0	9 (81.8)		1 (9.1)	1 (9.1)	
TILs							
Present	32	2 (6.3)	17 (53.1)	>.99	11 (34.3)	2 (6.3)	>.99
Absent	28	2 (7.1)	19 (67.9)		6 (21.4)	1 (3.6)	
Tumor necrosis							
Present	26	2 (7.7)	16 (61.5)	>.99	6 (23.1)	2 (7.7)	.537
Absent	34	2 (5.8)	20 (58.9)		11 (32.4)	1 (2.9)	
Overall survival							
Alive	25	4 (16.0)	4 (16.0)	<.001	17 (68.0)	0	<.001
Dead	35	0	32 (91.4)		0	3 (8.6)	

Values are presented as number (%).

All MLAC cases with pleural involvement are classified as stage IV (M1a) disease. The T category refers to the size and extent of the primary lung tumor in the lung.

TLR9, Toll-like receptor 9; MLAC, metastatic lung adenocarcinoma; TIL, tumor-infiltrating lymphocyte.

p-value < .05 is considered statistically significant.

Table 3. Multivariate survival analysis in pleural malignancy cases

	Hazard ratio	95% CI	p-value
Age	0.982	0.950–1.015	.280
Sex	1.665	0.944–2.936	.078
Inflammation	0.744	0.394–1.404	.361
Necrosis	0.609	0.335–1.108	.104
Grade	1.202	0.543–2.663	.650
T category	0.930	0.570–1.519	.773
TLR9 expression	6.989	2.148–22.738	.001

Test of significance: Cox regression test.

CI, confidence interval; TLR9, Toll-like receptor 9.

p < .05 is considered statistically significant.

tumorigenesis. Stimulation of TLR9 and high TLR9 expression were linked to tumor hallmarks in several cancers, including lung cancer [13,14], prostate cancer [15], hepatocellular carcinoma

[16], and breast cancer [17]. Furthermore, previous studies underscored the role of TLR9 in tumorigenesis and cancer cell survival, through its effects on the activation of anti-apoptotic molecules such as Bcl2 and XIAP [18].

In contrast to our results, Droemann et al. [19] and Zhang et al. [14] found that TLR9 expression was significantly high in lung adenocarcinoma cases. However, the mentioned expression in their studies was observed in primary lung tumors, and no data were provided regarding its expression in metastatic pleural deposits of lung adenocarcinoma, as is the case with our findings. The discrepancy in receptors expression between primary tumors and metastases was reported in previous studies. For instance, a study on breast cancer revealed that the estrogen receptor was highly expressed in primary tumors whereas weak expression was observed in metastases [20]. Another study

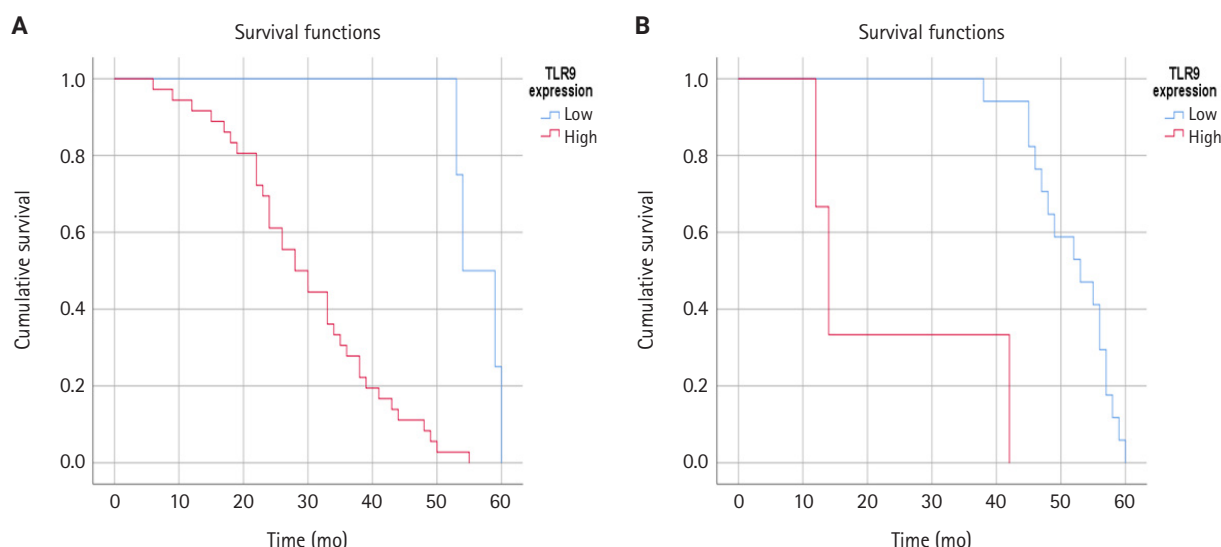


Fig. 3. The relationship between Toll-like receptor 9 (TLR9) expression and overall survival in mesothelioma (A) and metastatic lung adenocarcinoma (MLAC) (B). Kaplan-Meier curves showing that high TLR9 expression is associated with shorter overall survival in patient with pleural epithelioid mesothelioma and not in MLAC.

showed that connexin 26 and 43 decreased in primary tumors compared to metastatic tumors [21]. This heterogeneity of receptors expression is a well-known phenomenon in cancer biology [19]. While primary tumors may benefit from high TLR9 expression, metastatic cells might downregulate TLR9 to evade immune detection in new sites, which could explain the reduction of TLR9 in MLAC that was detected in our study.

Interestingly, a significant positive association was detected between TLR9 expression and T category of tumor in mesothelioma, whereas an inverse relationship was observed in MLAC cases. This suggests that high TLR9 expression may be associated with tumor progression and spread in mesothelioma but its role in MLAC spread cannot be determined due to the lack of primary tumor expression values. Moreover, TLR9 receptor itself may elicit different functions depending on the tumor context. For instance, Gonzalez-Reyes et al. [15] observed that increased TLR9 expression correlated with greater aggressiveness in prostate cancer, while in other studies elevated TLR9 was linked to advanced stages.

In contrast, Singh et al. [22] reported that high TLR9 expression following chemotherapy showed evidence of antitumor activity. These discrepancies in TLR9 expression suggest that TLR9 expression may be changed with different tumor status, treatment context, or experimental design. It is also worth noting that in our study, TLR9 expression in MLAC was evaluated only in metastatic deposits, and not in primary tumors, which

may explain the lack of correlation with metastasis.

Survival analysis reported that high TLR9 expression in epithelioid mesothelioma was significantly associated with shorter OS ($p = .001$). TLR9 was suggested to be an independent prognostic factor by using multivariate Cox regression analysis (HR, 6.989; 95% CI, 2.148 to 22.738). This finding is comparable with other studies that revealed elevated TLR9 levels correlate with poor prognosis [23]. Conversely, no significant association between TLR9 expression and OS was observed in MLAC cases.

Interestingly, high TLR9 expression was detected in inflammatory cells in few cases of epithelioid mesothelioma (10%) and the tumor cells in these cases also revealed high TLR9 expression (Fig. 2). This suggests that TLR9 may have an additional action depending on its site, whether in tumor cells or in inflammatory cells [24].

Limitations of the study

First, the sample size, while sufficient for initial comparisons, is relatively small for robust survival and subgroup analyses. This limited statistical power likely explains why established prognostic factors like tumor stage did not reach significance in our multivariate model and precluded more sophisticated analyses to draw more definite conclusions. Therefore, our primary finding—that TLR9 expression is significantly higher in mesothelioma compared to MLAC—requires validation in a larger, independent cohort. Second, all cases were collected from a sin-

gle institution; future multi-center studies would enhance the generalizability of the results. Third, we were unable to perform Western blot analysis on the exact same fresh-frozen samples used for qRT-PCR due to insufficient tissue material; however, our immunohistochemical findings on a larger, independent cohort provide strong complementary validation at the protein level. Finally, this study did not explore the mechanistic role of TLR9 in mesothelioma pathogenesis, which remains a crucial area for future investigation.

Conclusions

To conclude, this study underscores the role of TLR9 in epithelioid mesothelioma. TLR9 shows potential as a complementary diagnostic marker to differentiate epithelioid mesothelioma from MLAC and a potentially prognostic biomarker due to its association with poor prognostic parameters in epithelioid mesothelioma. Furthermore, this study suggests that targeting TLR9 could be a potential therapeutic strategy.

Supplementary Information

The Data Supplement is available with this article at <https://doi.org/10.4132/jptm.2026.04.01>.

Ethics Statement

The study performed was approved by the Research Ethics Committee of the Faculty of Medicine at Minia University (approval No. 334-6-2022). Informed consent was obtained from all individual participants included in the study.

Availability of Data and Material

All data generated or analyzed during the study are included in this published article (and its supplementary information files).

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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The impairment of CD3⁺ T lymphocytes in sentinel lymph node of high-risk cutaneous melanoma patients in stage IIB and IIC

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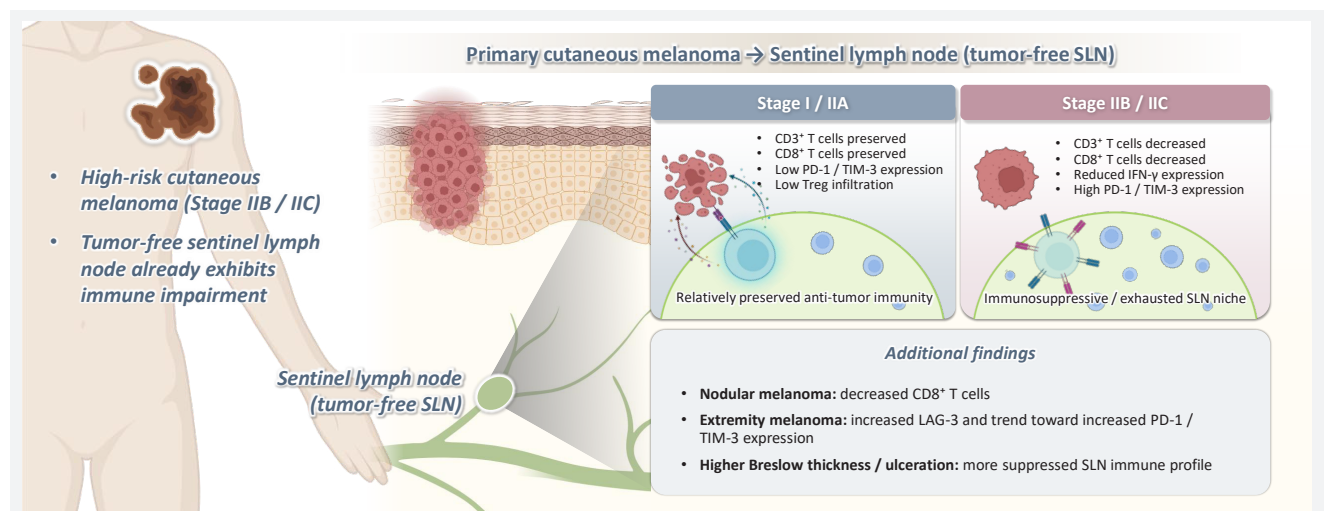
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Graphical abstract



CONCLUSION

This study provides new results of the impairment of immune response in SLN of cutaneous melanoma patients with high risk for metastasis and could help into the introduction of new immunotherapies that could revive immunity and prevent metastasis in SLN.

The impairment of CD3⁺ T lymphocytes in sentinel lymph node of high-risk cutaneous melanoma patients in stage IIB and IIC

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Background: The sentinel lymph node (SLN) in melanoma is almost always the first site of metastasis and its histopathological assessment is essential for the determination of staging and clinical outcome. Furthermore, this procedure offers the investigation of the early immune response in SLN as melanoma-derived factors suppress the immunity in an early stage that may facilitate metastasis. A better understanding of the immunological changes in SLN may help in the therapeutic stimulation of melanoma immunity to prevent tumor metastasis. **Methods:** SLN tissues without malignant cells from 74 cutaneous melanoma patients (stage I and II) were analyzed. By flow cytometry, we measured the percentage of natural killer cells, CD3⁺ T lymphocytes, and their expression of interferon- γ (IFN- γ) and inhibitory immune checkpoint molecules (ICMs), and the percentage of CD4⁺Foxp3⁺ regulatory T cells (Tregs). **Results:** Melanoma patients with worse prognosis, in stage IIB–C, had decreased percentage of total CD3⁺ and CD3⁺CD8⁺ T lymphocytes, trend of IFN- γ decrease, increased inhibitory programmed cell death 1 and T cell immunoglobulin and mucin-domain containing 3 ICMs, and higher percentage of Tregs in their SLNs compared with stage I-IIA patients. Furthermore, patients with nodular melanoma had decreased CD3⁺CD8⁺ cells compared with patients with superficial spreading melanoma and together with patients with localization of primary tumor on extremities had an increase in the expression of analyzed ICMs. **Conclusions:** This study provides new results of the impairment of immune response in SLN of cutaneous melanoma patients with high risk for metastasis and could help in the introduction of new immunotherapies that could restore immunity and prevent metastasis in SLN.

Keywords: Cutaneous malignant melanoma; Sentinel lymph node; T-Lymphocytes; Immune checkpoint proteins; Immunotherapy

INTRODUCTION

Melanoma is the most severe type of skin cancer, originating from melanocytes, the cells that produce melanin. This cancer primarily affects the skin, but melanoma may rarely develop in the mouth, intestines, or eye. Although it is a rare cancer, the incidence of melanoma has continuously increased over the last 50 years [1]. Although melanoma cells spread rapidly from the

primary tumor to the distant organs through the bloodstream, tumor cells often use the lymphatic system for metastatic progression [2].

The sentinel lymph node (SLN) is the first lymph node to receive drainage directly from a tumor and is almost always the first site of melanoma metastasis. Histopathological assessment of SLN for the presence of metastatic cells is essential for melanoma staging and its results may represent a key predictor of

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disease outcome, recommended by the National Comprehensive Cancer Network [3]. Additionally, this procedure offers an excellent opportunity to investigate the early immune response to tumor antigens in SLN.

It is known that lymph nodes (LNs) as secondary lymphoid tissue are the foci of lymphocytes. Under physiological conditions, they play a central role in immunosurveillance of non-self-antigens to which they are exposed via afferent lymph. Therefore, SLNs are critical sites of antigen presentation and the activation of the cells of adaptive immune system against tumor [4]. However, SLN is also the first tumor-draining LN exposed to inhibitory factors secreted from primary tumor [5]. These mediators including cytokines (interleukin [IL]-1 β , IL-6, IL-8, IL-10, transforming growth factor β [TGF- β]), chemokines (CXCL1, CXCL2, CXCL8, and CCL2), growth factors (vascular endothelial growth factor [VEGF]), as well as enzyme indoleamine 2,3-dioxygenase enable cancer cell-mediated evasion of immunosurveillance and can serve as immunosuppressants, facilitating melanoma cells to invade LNs. These factors play the role in the differentiation, proliferation and activation of numerous immunosuppressive cells such as myeloid-derived suppressor cells, regulatory T cells (Tregs), tumor-associated macrophages that play an important role in tumor growth and metastasis by inhibiting anti-tumor activity of CD3⁺CD4⁺ helper T (Th) cells, CD3⁺CD8⁺ cytotoxic T lymphocytes (CTLs), natural killer (NK) cells [5,6]. These inhibitory factors also alter the functions of dendritic cells (DCs) within the SLN, leading to failures in tumor antigen presentation and T-cell activity [7]. These processes promote the maintenance of an immunosuppressive microenvironment, so-called premetastatic niche (PMN), in SLN and favor early metastasis in SLN [5,6].

Immunomodulators and blockers of inhibitory melanoma-secreted mediators are under clinical investigation for the prevention of lymphatic metastasis as they interrupt PMN development. Understanding the mechanisms of PMN formation offers an opportunity for developing new therapeutic approaches that could prevent melanoma metastasis in high-risk melanoma patients with local, cutaneous tumor [8]. Therefore, new immune therapies, immune checkpoint inhibitors (ICIs), monoclonal antibodies (mAbs) that block inhibitory molecules that are highly expressed on the surface of immune and tumor cells in tumor microenvironment (TME) such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1), lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and

mucin-domain containing 3 (TIM-3) restore the immune system by increasing T and NK cell-mediated anti-tumor activity. Therefore, ICIs could enable tumor regression and long-term cancer control in melanoma patients [9].

There is little data in the literature on the immunophenotypic and functional characteristics of lymphocytes in still intact, non-metastatic SLN in patients with cutaneous, localized melanoma. For this reason, this research should provide completely new data related to the potential difference in the percentage and function of T lymphocytes and their subsets, as well as the expression of inhibitory molecules, PD-1, LAG-3, and TIM-3 on their surface between patients in clinical stage II with a worse prognosis and higher possibility of metastasis compared with the patients in clinical stage I. Although, a few years ago, the Food and Drug Administration (FDA) approved the application of anti-PD-1 mAbs, pembrolizumab and nivolumab, for adjuvant therapy in high-risk melanoma patients in stage IIB or IIC [10], it remains an open question whether all these patients are candidates for treatment with this therapy, considering its potential side effects and possible resistance.

The results of this research could contribute to a more precise selection of patients in whom this therapy would give the best clinical response. Furthermore, the results related to the expression of not only PD-1, but also the less analyzed LAG-3 and TIM-3 inhibitory molecules on the surface of SLN lymphocytes could show their importance in reducing the function of T lymphocytes, as well as the potentially great importance of their therapeutic blockade in patients with a high risk of metastasis in stage II of melanoma.

MATERIALS AND METHODS

Patients and SLN analysis

In this prospective study, we analyzed the samples of SLN tissue from 80 patients with cutaneous melanoma, aged over 18 years, regardless of their race, sex, or ethnic origin, who underwent the biopsy of SLN at the Institute of Oncology and Radiology of Serbia (IORS). The research was ongoing from April 2024 to August 2025. For diagnostic purposes, SLNs are routinely analyzed for the presence of melanoma cells in patients who have melanomas with Breslow thickness ≥ 0.8 mm or thinner but with the presence of ulceration (stage T1b and higher according to the TNM tumor classification), and do not have clinically and diagnostically observed signs of pathological lymphadenopathy and/or metastatic changes [11]. Patients under the age

of 18, as well as patients with infectious, autoimmune diseases, acute or chronic inflammation of the other origin, were excluded from the research. For the purpose of SLN checking, preoperatively, a marker, a radioactive isotope Tc99m was injected intradermally into the region of the scar from the removed melanoma, and immediately preoperatively another marker, a vital dye (methylene blue), was injected in the same way. During the surgical intervention, a lymph node that received one or both markers was removed from the regional lymph pool, which is considered to be the SLN. SLNs are either positive or negative for melanoma cells based on the pathohistological analysis of serial sections by hematoxylin and eosin and immunohistochemical staining. In the case of a positive SLN, patients are classified in clinical stage III of the disease, and in the case of a negative SLN in stage I or II, depending on the thickness of the primary melanoma and the presence of ulceration. A random section of SLN tissue (maximum 1 mm thick) from each patient, obtained fresh by the pathologist immediately after surgery, was transferred to cryovials and cryopreserved at -150°C for storage and later analysis. As only six patients had metastasis in their SLNs (stage III), the aim of this study was to analyze the immunological parameters of 74 patients with melanoma cell-negative SLNs (clinical stage I and II). The characteristics of patients in clinical stages I and II and their primary tumors are included in Table 1.

Mononuclear cell isolation

In order to form single cell suspension, SLN tissue samples were mechanically minced with sterile scalpel and filtered through a 100 μm mesh to exclude undissociated fragments. Mononuclear cells (MNCs), lymphocytes, and monocytes, were isolated using Histopaque (Sigma-Aldrich Chemie, Steinheim, Germany) density gradient, centrifuged at 1,600 rpm for 40 minutes and washed three times in RPMI 1640 cell culture medium (CM) supplemented with 10% fetal calf serum (Sigma-Aldrich).

Flow cytometric analysis

The percentage of total T lymphocytes and their different subpopulations, the percentage of NK cells, as well as the expression of inhibitory receptors, PD-1, LAG-3, TIM-3, on the surface of total T cells in the lymphocyte population in freshly isolated MNCs originating from SLNs of patients with skin melanoma were determined by the method of direct three-color immunofluorescence on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) using mAbs marked with

fluorescent dyes. Samples for analysis were prepared according to the method of Jackson and Warner [12]. Namely, 5×10^5 freshly isolated MNCs in a suspension of 100 μL (concentration 5×10^6 cells/mL) were incubated for 30 minutes at 4°C with 10 μL of the appropriate combination of mAbs and then washed twice by centrifugation at 1,600 rpm in cold phosphate buffer solution (PBS) (CellWASH, Becton Dickinson) and for analysis on a flow cytometer resuspended in 1% paraformaldehyde (CellFIX, Becton Dickinson).

In the suspension of peripheral blood lymphocytes and

Table 1. The patients and their primary tumor characteristics

Characteristics of patients in stage I and II	No. (%)
Sex	
Male	42 (56.8)
Female	32 (43.2)
Age (yr)	
Mean $\pm$ SD	61.0 $\pm$ 14.6
Median (range)	66 (21–88)
Stage of disease	
I	30 (40.5)
IIA	15 (20.3)
IIB	15 (20.3)
IIC	14 (18.9)
Tumor characteristics	
Breslow	
≤ 2 mm	35 (47.3)
> 2 mm	39 (52.7)
Ulceration	
No	43 (58.1)
Yes	31 (41.9)
Tumor infiltrating lymphocytes	
Non-brisk	23 (31.1)
Brisk	41 (55.4)
Unknown	10 (13.5)
Histologic subtypes	
SSM	38 (51.3)
NM	21 (28.4)
ALM	1 (1.3)
Unknown	14 (19.0)
Localization	
Extremities	37 (50.0)
Trunk	36 (48.6)
Head	1 (1.4)
Total	74 (100)

SD, standard deviation; SSM, superficial spreading melanoma; NM, nodular melanoma; ALM, acral lentiginous melanoma.

monocytes, about 20,000 cells per sample were collected and verified as lymphocytes according to their physical characteristics: size (forward scatter) and granularity (side scatter), gated and analyzed using CellQUEST software. The degree of non-specific immunofluorescence was determined using matched isotype-identical, IgG1 mAbs controls (Becton Dickinson). In more detail, according to convention, we used the 1% level to separate negative and positive cells. The markers were placed to delineate a region of positive intensity maximum 1% of negative control cells.

The following combinations of mAbs labeled with fluorescent dyes were used: CD8FITC/CD4PE/CD3APC, CD56FITC/PD-1PE/CD3APC, LAG-3FITC/TIM-3PE/CD3APC, CD25FITC/Foxp3PE/CD4PerCP (Becton Dickinson). Total T lymphocytes were detected and analyzed as CD3⁺ lymphocytes, Th lymphocytes as CD3⁺CD4⁺, CTLs as CD3⁺CD8⁺, NK cells as CD3⁺CD56⁺, and NKT-like cells as CD3⁺CD56⁺ lymphocytes. The expression in percentage of PD-1, LAG-3, and TIM-3 molecules was determined on the surface of total CD3⁺ T lymphocytes.

For the analysis of the percentage of Tregs within the SLN tissue by the flow cytometry method, a sample of 1×10^6 MNCs isolated from the SLN tissue of melanoma patients was prepared and resuspended in 100 μ L of CM. External antigens that determine the immunophenotype of Treg cells were labeled with 10 μ L of mAbs conjugated with fluorescent dyes, CD-25FITC and CD4PerCP (Becton Dickinson) and incubated for 30 minutes at 4°C. After that, the samples were washed twice by centrifugation at 1,600 rpm in a cold PBS. After the staining of external antigens, according to the standard procedure of permeabilization, the MNCs were exposed to the BD FACS solution 2 for permeabilization of cells containing the detergent saponin (Perm 2, BD Biosciences) and after that, for the labeling of intracellular Foxp3, 10 μ L of anti-Foxp3 PE monoclonal antibody (Becton Dickinson) was added to each sample. After two washes in cold PBS, samples were dissolved in 1% paraformaldehyde for flow cytometer analysis. In the lymphocyte population of SLN, the percentage of Tregs was analyzed as the percentage of Foxp3⁺ and CD4⁺Foxp3⁺ cells.

Interferon- γ measurement

The flow cytometry method was used to determine the expression of synthesized interferon- γ (IFN- γ) in CD3⁺ T cells. A sample of 5×10^5 MNCs isolated from SLN tissue of a melanoma patient was prepared and resuspended in 500 μ L CM. MNCs prepared in this way were cultured at a temperature of

37°C in polystyrene test tubes for flow cytometry with 50 ng/mL phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) and 500 ng/mL ionomycin (Sigma-Aldrich), which have the role to stimulate the synthesis of IFN- γ . After 1 hour of incubation with PMA and ionomycin, 10 mg/mL brefeldin A (Sigma-Aldrich) was added to prevent the transport of synthesized proteins from the cells into the external environment. After an additional 3 hours (a total of 4 hours of treatment), 10 μ L of a monoclonal antibody conjugated with a fluorescent dye, CD3 FITC (Becton Dickinson), was added to the MNCs and the labeled MNCs were incubated for 30 minutes at 4°C. After that, the samples were washed twice by centrifugation at 1,600 rpm in a cold PBS. After external antigen staining, according to the standard procedure for permeabilization, the MNC sediment was exposed to Perm 2 solution and then 10 μ L of anti-IFN- γ PE monoclonal antibody (Becton Dickinson) was added to each sample to label intracellular IFN- γ . After two washes in cold PBS, the samples were dissolved in 1% paraformaldehyde. In the lymphocyte population of the SLN tissue, the percentage of CD3⁺ T cells containing IFN- γ was determined.

Statistical analysis

The obtained results were analyzed using the non-parametric test, the Mann-Whitney exact test of unpaired data by the GraphPad Prism program (GraphPad Software Inc., San Diego, CA, USA). Correlations between data were determined by Spearman's rank correlation. The differences between the compared groups were statistically significant or there was a correlation between investigated parameters if the calculated p-value was less than or equal to .05.

RESULTS

The percentage of T and NK cells

In this study, we analyzed the percentage of T lymphocyte subsets and NK cells in the samples of SLN tissue from 74 patients with cutaneous, localized melanoma in clinical stages I and II. We have found that patients in stage II and especially in stages IIB and IIC had statistically significant decrease ($p \leq .05$ and $p < .01$, Mann-Whitney exact test) in the percentage of total CD3⁺ and cytotoxic CD3⁺CD8⁺ T cells compared with the percentage of these cells in the patients in stage I, as well as in stage I and stage IIA together (Fig. 1A–C). However, there was no difference ($p > .5$, Mann-Whitney exact test) in the percentage of helper CD3⁺CD4⁺ T cells, as well as CD3⁺CD56⁺ NK

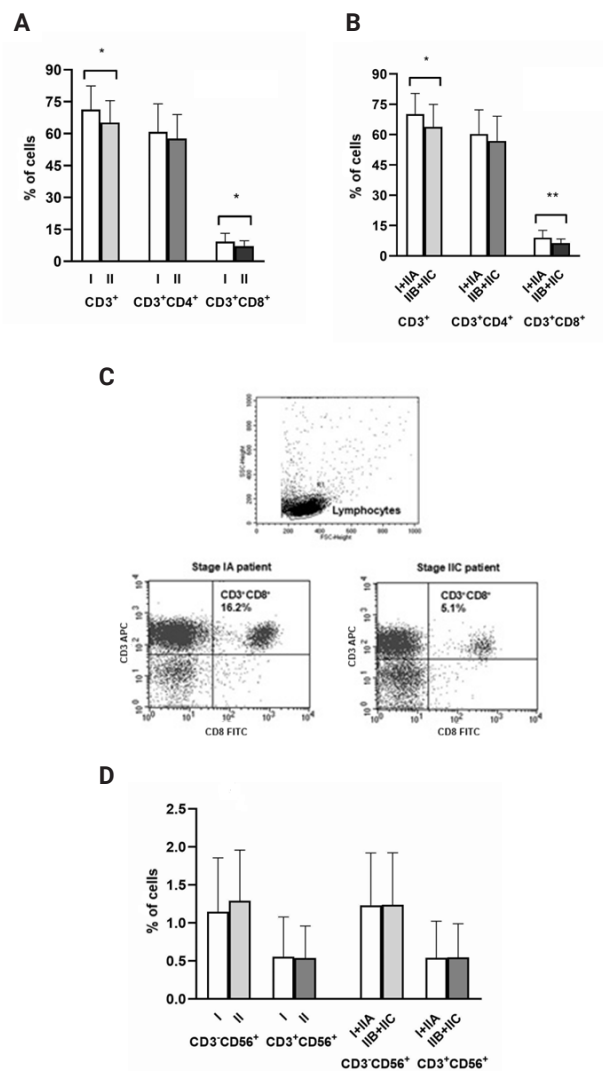


Fig. 1. The percentage of T and natural killer cells. (A) The patients in stage II compared with the patients in stage I have a statistically significant decrease in the percentage of total CD3⁺, as well as cytotoxic CD3⁺CD8⁺ T cells ($p \leq .05$, Mann-Whitney exact test). (B) The patients in stage IIB + IIC compared with the patients in stage I + IIA have a statistically significant decrease in the percentage of total CD3⁺ ($*p \leq .05$) and a highly significant decrease ($**p < .01$) in the percentage of cytotoxic CD3⁺CD8⁺ T cells (Mann-Whitney exact test). (C) Representative dot plots of decreased percentage of CD3⁺CD8⁺ T cells in a melanoma patient in stage IIC compared with a patient in stage IA. (D) There is no difference ($p > .5$, Mann-Whitney exact test) in the percentage of CD3⁺CD56⁺ NK and CD3⁺CD56⁺ NKT-like cells between all investigated groups of melanoma patients (stage I vs. stage II, as well as stage I + IIA vs. stage IIB + IIC). The results are obtained from the samples of sentinel lymph node tissue of 74 patients with cutaneous melanoma and presented as mean value with standard error.

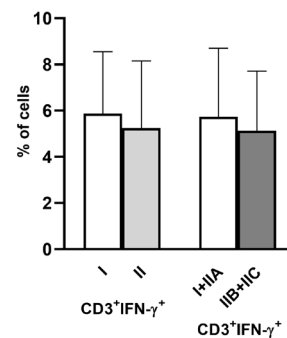


Fig. 2. The expression of interferon γ (IFN- γ) in T cells. There is no statistically significant difference ($p > .5$, Mann-Whitney exact test) in the percentage of IFN- γ in CD3⁺ T cells between melanoma patients in stage I and stage II, as well as in stage I + IIA and stage IIB + IIC. The results are obtained from the samples of sentinel lymph node tissue of 50 patients with cutaneous melanoma and presented as mean value with standard error.

and CD3⁺CD56⁺ NKT-like cells between investigated groups of melanoma patients (Fig. 1A, B, D).

The expression of IFN- γ in T cells

Analyzing the expression of IFN- γ in T cells of SLNs, we have shown that there was no statistically significant difference ($p > .5$, Mann-Whitney exact test) in the percentage of this cytokine in CD3⁺ T cells between melanoma patients in stage I and stage II, as well as in stage I + IIA and stage IIB + IIC. However, there was a trend of decreased expression of IFN- γ in CD3⁺ T cells between the analyzed groups of melanoma patients (Fig. 2).

The expression of PD-1, TIM-3, and LAG-3 on T cells

Analyzing the expression of inhibitory molecules PD-1, TIM-3, and LAG-3 on T cells in SLN tissue we have found that the percentage of PD-1 molecule was significantly higher ($p < .01$, Mann-Whitney exact test) in melanoma patients in stage II compared with patients in stage I (Fig. 3A). Additionally, melanoma patients in stage IIB and IIC, besides significantly higher expression of PD-1 molecule ($p < .01$, Mann-Whitney exact test), had significantly increased expression of the other inhibitory molecule, TIM-3 ($p \leq .05$, Mann-Whitney exact test) on CD3⁺ T cells compared with the patients in stage I and IIA (Fig. 3B, C). However, analyzing the expression of all analyzed inhibitory molecules on T cells in SLNs in patients in stage IIB and IIC, as well as in patients in stage III ($n = 6$), we found no difference ($p > .5$, Mann-Whitney exact test) in their values (Fig. 3D).

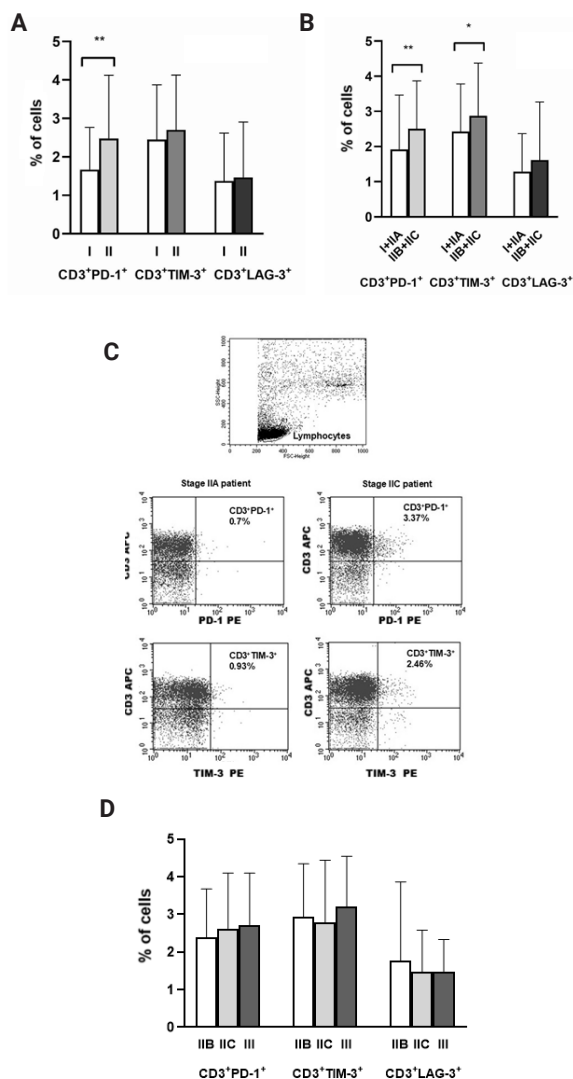


Fig. 3. The expression of PD-1, TIM-3, and LAG-3 on T cells. (A) The percentage of PD-1 molecule on CD3⁺ T cells is significantly higher (** $p < .01$, Mann-Whitney exact test) in melanoma patients in stage II compared with the patients in stage I. (B) Melanoma patients in stage IIB + IIC have significantly higher expression of PD-1 (** $p < .01$), as well as TIM-3 molecule (* $p \leq .05$) on CD3⁺ T cells compared with the patients in stage I-IIA (Mann-Whitney exact test). (C) Representative dot plots of increased percentage of CD3⁺PD-1⁺ and CD3⁺TIM-3⁺ T cells in a melanoma patient in stage IIC compared with a patient in stage IIA. (D) There is no difference ($p > .5$, Mann-Whitney exact test) in the expression of PD-1, TIM-3, and LAG-3 inhibitory molecules on CD3⁺ T cells between melanoma patients in stage IIB, stage IIC, as well as in stage III. The results are obtained from the samples of sentinel lymph node tissue of 74 patients with cutaneous melanoma in stages I and II and six patients in stage III and presented as mean value with standard error. PD-1, programmed death 1; TIM-3, T cell immunoglobulin and mucin-domain containing 3; LAG-3, lymphocyte activation gene-3.

The percentage of Tregs

In melanoma patients in stage I and stage II, we also analyzed the percentage of Tregs in their SLNs. We have shown that there was no statistically significant difference ($p > .5$, Mann-Whitney exact test) in the percentage of Foxp3⁺ and CD4⁺ Foxp3⁺ Tregs between melanoma patients in stage I and stage II (Fig. 4A). However, melanoma patients in stage IIB and IIC had significantly higher percentage ($p \leq .05$, Mann-Whitney exact test) of Foxp3⁺ and CD4⁺ Foxp3⁺ Treg cells in SLNs compared with the percentage of these cells in stage I and IIA (Fig. 4A, B).

The association between the percentage of immune cells in SLN and different histological characteristics of primary melanoma

Analyzing the association between the percentage of immune cells in SLNs and histological characteristics of primary melanoma, we found a negative correlation ($p \leq .05$, Spearman rank correlation) between primary tumor thickness (Breslow) and the percentage of total CD3⁺, as well as CD3⁺CD8⁺ T cells in SLNs of investigated melanoma patients. Contrary to these results, we showed a positive correlation ($p \leq .05$, Spearman rank correlation) between Breslow thickness and the percentage of CD3⁺PD-1⁺ T lymphocytes (Fig. 5A). Furthermore, the patients with ulceration in primary tumor had statistically significant decrease ($p \leq .05$ and $p < .01$, Mann-Whitney exact test) in the percentage of CD3⁺ and CD3⁺CD8⁺ T cells, as well as significant increase ($p \leq .05$, Mann-Whitney exact test) in the percentage of CD3⁺PD-1⁺ cells compared with the percentage of these cells in the patients without primary tumor ulceration. However, there was no difference ($p > .5$, Mann-Whitney exact test) in the percentage of CD3⁺TIM-3⁺ and CD3⁺LAG-3⁺ cells between investigated groups of melanoma patients (Fig. 5B). On the other hand, there was no statistically significant difference ($p > .5$, Mann-Whitney exact test) in the percentage of analyzed immune cells (CD3⁺ and CD3⁺CD8⁺ T cells), as well as in the expression of inhibitory PD-1, TIM-3, and LAG-3 molecules on CD3⁺ T cells between patients with focal (non-brisk) and dense (brisk) lymphocyte infiltrate in their primary tumors (Fig. 5C).

The association between the percentage of immune cells in SLN and different histological subtypes and localizations of primary melanoma

Making a comparison in immune cells in SLNs between melanoma patients with two different histologic subtypes, we have found that patients with nodular melanoma (NM) ($n = 21$)

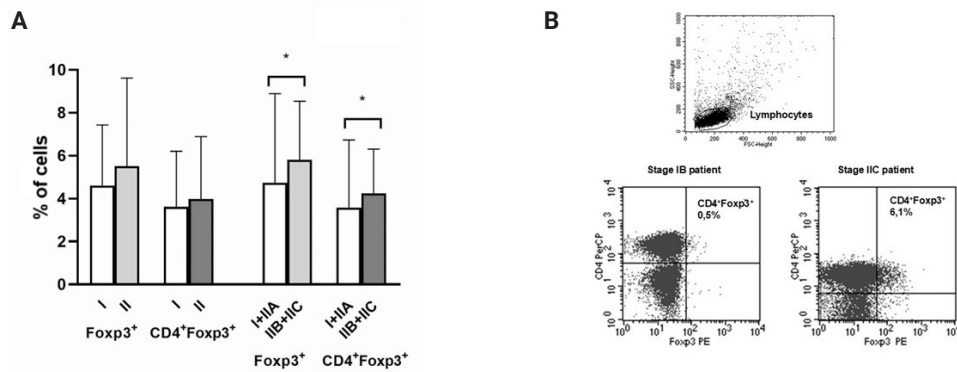


Fig. 4. The percentage of regulatory T cells (Tregs). (A) There is no statistically significant difference ($p > .05$, Mann-Whitney exact test) in the percentage of Fcpx3⁺ and CD4⁺Fcpx3⁺ Tregs between melanoma patients in stage I and stage II, whereas melanoma patients in stage IIB + IIC have significantly higher percentage of Fcpx3⁺ and CD4⁺Fcpx3⁺ Treg cells compared with the percentage of these cells in stage I + IIA (* $p \leq .05$, Mann-Whitney exact test). (B) Representative dot plots of increased percentage of Fcpx3⁺ and CD4⁺Fcpx3⁺ Treg cells in a melanoma patient in stage IIC compared with a patient in stage IB; the results are obtained from the samples of sentinel lymph node tissue of 65 patients with cutaneous melanoma and presented as mean value with standard error.

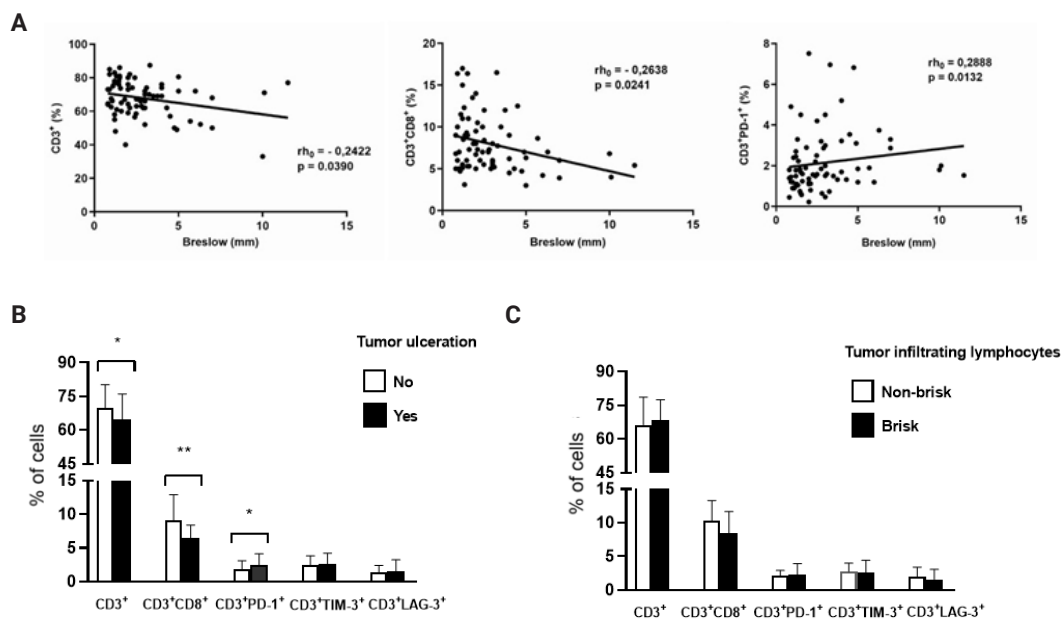


Fig. 5. The association between the percentage of immune cells in sentinel lymph node (SLN) and different histological characteristics of primary melanoma. (A) The negative correlation ($p \leq .05$, Spearman rank correlation) between primary tumor thickness in millimeters (Breslow) and the percentage of total CD3⁺, as well as CD3⁺CD8⁺ T cells, as well as a positive correlation ($p \leq .05$, Spearman rank correlation) between Breslow thickness and the percentage of CD3⁺PD-1⁺ T lymphocytes in SLN of all investigated melanoma patients are found. (B) The patients with ulceration in primary tumor have a statistically significant decrease in the percentage of CD3⁺ (* $p \leq .05$) and CD3⁺CD8⁺ (** $p < .01$) T cells, as well as significant increase ($p \leq .05$) in the percentage of CD3⁺PD-1⁺ cells compared with the percentage of these cells in the patients without primary tumor ulceration (Mann-Whitney exact test). There is no difference ($p > .05$, Mann-Whitney exact test) in the percentage of CD3⁺TIM-3⁺ and CD3⁺LAG-3⁺ cells between the investigated groups of melanoma patients. (C) There is no statistically significant difference ($p > .05$, Mann-Whitney exact test) in the percentage of CD3⁺ and CD3⁺CD8⁺ T cells, as well as in the expression of inhibitory PD-1, TIM-3, and LAG-3 molecules on CD3⁺ cells between patients with focal (non-brisk) and dense (brisk) lymphocyte infiltrate in their primary tumors. The results are obtained from the samples of SLN tissue of a maximum of 74 patients. PD-1, programmed death 1; TIM-3, T cell immunoglobulin and mucin-domain containing 3; LAG-3, lymphocyte activation gene-3.

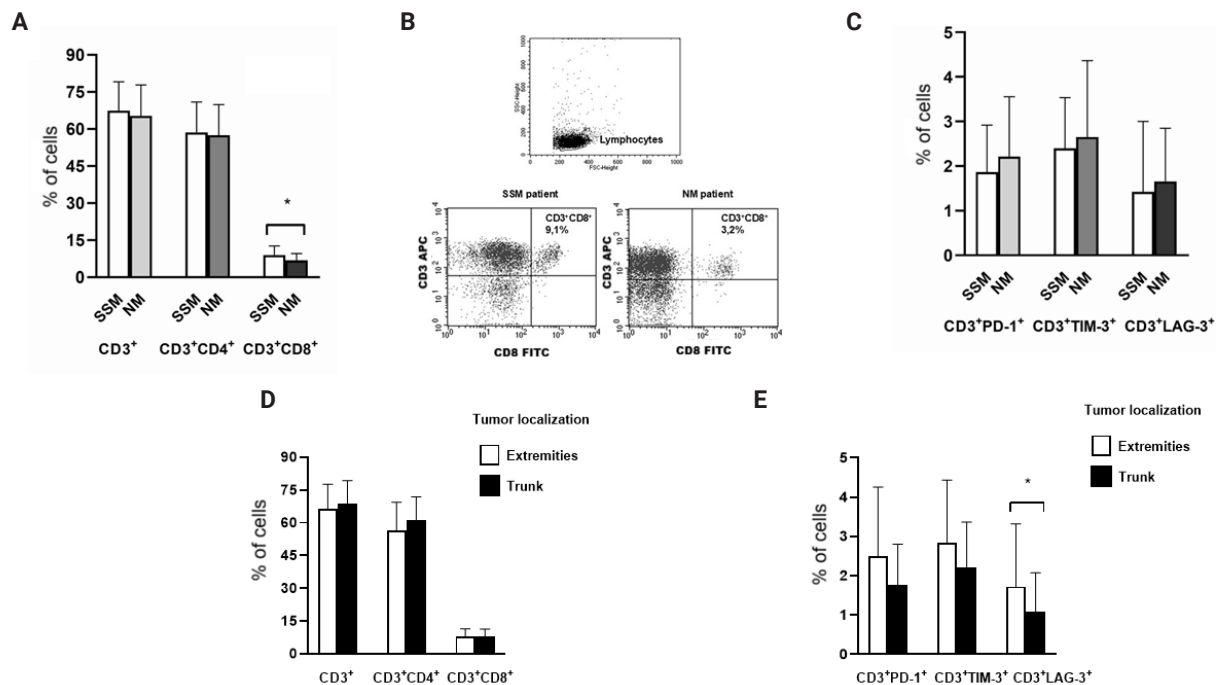


Fig. 6. The association between the percentage of immune cells in sentinel lymph node (SLN) and different histological subtypes and localizations of primary melanoma. (A) The patients with nodular melanoma (NM) ($n = 21$) have a statistically significant decrease ($*p \leq .05$, Mann-Whitney exact test) in the percentage of cytotoxic CD3⁺CD8⁺ T cells compared with the percentage of these cells in the patients with superficial spreading melanoma (SSM) ($n = 38$). There is no statistically significant difference ($p > .05$, Mann-Whitney exact test) in the percentage of total CD3⁺ and CD3⁺CD4⁺ T cells between melanoma patients with SSM and NM. (B) Representative dot plots of decreased percentage of CD3⁺CD8⁺ T cells in an NM patient compared with a patient with SSM histological subtype. (C) No statistically significant difference ($p > .05$, Mann-Whitney exact test) is found in the expression of PD-1, TIM-3, and LAG-3 inhibitory molecules on CD3⁺ T cells in SLNs of SSM and NM patients. (D) The percentages of CD3⁺, CD3⁺CD8⁺, and CD3⁺CD4⁺ T cells in SLNs do not significantly differ ($p > .05$, Mann-Whitney exact test) in cutaneous melanoma patients with primary localization of tumor on extremities and trunk. (E) The expression of LAG-3 inhibitory molecule is significantly increased ($*p \leq .05$, Mann-Whitney exact test) on CD3⁺ T cells in SLNs of patients with primary melanoma localization on extremities compared with patients with tumor localization on trunk. There is no significant difference ($p > .05$, Mann-Whitney exact test) in the expression of PD-1 and TIM-3 on CD3⁺ cells among these two groups of melanoma patients. The results are obtained from the samples of SLN tissue of maximum 74 patients with cutaneous melanoma and presented as mean value with standard error. PD-1, programmed death 1; TIM-3, T cell immunoglobulin and mucin-domain containing 3; LAG-3, lymphocyte activation gene-3.

had statistically significant decrease ($p \leq .05$, Mann-Whitney exact test) in the percentage of cytotoxic CD3⁺CD8⁺ T cells compared with the percentage of these cells in the patients with superficial spreading melanoma (SSM) ($n = 38$) (Fig. 6A, B). Additionally, analyzing the expression of PD-1, TIM-3, and LAG-3 molecules on CD3⁺ T cells in SLN tissues of SSM and NM patients, we found no statistically significant difference ($p > .05$, Mann-Whitney exact test) in the expression of all analyzed inhibitory molecules between these two histologic subtypes of melanoma patients. However, there was a trend of increased expression of PD-1, TIM-3, as well as LAG-3 on CD3⁺ T cells in NM patients (Fig. 6C). Additionally, we analyzed the per-

centage of immune cells in SLNs in cutaneous melanoma patients with primary localization of tumor on extremities and trunk and found no statistically significant difference ($p > .05$, Mann-Whitney exact test) in the percentage of CD3⁺, cytotoxic CD3⁺CD8⁺ and helper CD3⁺CD4⁺ T cells between these two different tumor localizations (Fig. 6D). However, analyzing the expression of PD-1, TIM-3, and LAG-3 molecules on CD3⁺ T cells in SLNs, we found statistically significant increase ($p \leq .05$, Mann-Whitney exact test) of LAG-3 inhibitory molecule in patients with primary melanoma localization on extremities. Additionally, there was a trend of increased expression of PD-1 and TIM-3 on CD3⁺ cells in these patients (Fig. 6E).

DISCUSSION

Although it is well-known that melanoma cells spread rapidly to distant sites through both hematologic and lymphatic pathways, SLN is almost always the first site of melanoma metastasis. At the beginning of this process, the cells of primary tumor secrete various immunosuppressive soluble mediators [5], as well as extracellular vesicles (EVs) [13] that are transported through afferent lymphatic vessels to SLN. These factors are able to suppress anti-tumor immune response and induce remodeling and lymphangiogenesis in SLNs [6]. These processes occur before the arrival of the first metastatic cells in SLN and they have the role in the maintenance of PMN that helps in the colonization of metastatic cells in SLN [14].

In this study we have shown that melanoma patients with worse prognosis in stage II, and especially with thicker melanoma and the presence of ulceration (stage IIB and IIC) had statistically significant decrease in the percentage of total CD3⁺ and cytotoxic CD3⁺CD8⁺ T cells compared with the patients in stage I melanoma, a less aggressive stadium of the cancer disease with a lower risk of spreading. Grotz et al. [15] found fewer CTLs in SLN compared with the percentage of these cells in non-SLN and healthy LN of melanoma patients with cutaneous melanoma. Total CD3⁺, especially CD8⁺ lymphocytes, exhibit cytotoxic properties, promoting the removal of cancer cells indirectly by cytokines production (IFN- γ and tumor necrosis factor α), as well as directly through the secretion of cytotoxic proteins, perforin, and granzymes [16]. Soluble mediators such as TGF- β , IL-6, IL-10, and VEGF produced in immunosuppressive microenvironment of primary tumor in stage II melanoma patients are transported to SLN and induce impairment of the functions of CD8⁺ cytotoxic T cells [17]. Our group found increased serum level of the immunosuppressive cytokine TGF- β 1 in melanoma patients in stage IIB and IIC compared with the patients in stage IA and IB [18]. It is known that TGF- β suppresses the synthesis of perforin and granzymes, and also inhibits the ERK pathway, which is important for T-cell proliferation and cytokine production that leads to a loss of immune surveillance against melanoma [19]. In accordance with this knowledge, in this study, we found a trend of decreased expression of IFN- γ in CD3⁺ T cells in SLN of melanoma patients in stage II compared with patients in stage I. IFN- γ has the ability to inhibit tumor cell growth and metastasis directly through apoptosis and cell cycle arrest, as well as indirectly by the activation of anti-tumor immune cells, CTLs,

NK cells, and M1 macrophages [20]. Furthermore, EVs are associated with the loss of CTLs and impairment of their effector functions through high expression of the inhibitory PD-L1 molecule on their surface [21]. Therefore, melanoma patients in clinical stage II with a lower percentage of CD3⁺ and CTL CD8⁺ T lymphocytes and decreased IFN- γ production in still intact SLNs are at greater risk for tumor cell metastasis. Furthermore, although helper CD4⁺ T cells [22] and especially NK cells [23] play a significant role in anti-tumor immune response, in this study, we did not find any difference in the percentage of these cells in SLNs between melanoma patients in stage I and stage II. Some authors have shown diminished NK cells but only in melanoma-bearing SLNs [24].

The binding of T-cell inhibitory checkpoint molecules, PD-1, TIM-3, and LAG-3 to their ligands on tumor cells, as well as on numerous immunosuppressive cells in TME leads to T-cell dysfunction and exhaustion [25]. Also, it is known that PD-1-mediated T-cell failures begin early in melanoma progression [26]. Increased expression of PD-1 on T cells in SLN of stage II melanoma patients found in this study may suggest impairment in T-cell activation and function. Grotz et al. [15] showed increased expression of PD-1 on CD3⁺ T cells in SLN, as well as in non-SLN of cutaneous melanoma patients, compared with healthy LN [15]. Therefore, their SLNs develop an immune-tolerant microenvironment with altered immune cell profiles. Additionally, we show for the first time that melanoma patients in stage IIB and IIC, besides high expression of PD-1, had significantly increased expression of the other inhibitory molecule, TIM-3 on CD3⁺ T cells compared with the patients in stage I and IIA. TIM-3 is expressed on Th1, Th17, and CD8 T cells and by binding the ligands, galectin-9, phosphatidylserine, HMGB1, and CEACAM1, inhibits immune response of these T cells [27]. In physiological conditions, TIM-3 has a role in the maintenance of peripheral tolerance [28]. However, recent studies have shown an important role of TIM-3 molecule in T-cell exhaustion in cancer as TIM-3-expressing CD4⁺ and CD8⁺ T cells have impaired effector functions, are less proliferative in response to tumor antigens, and produce reduced amounts of cytokines that leads to failure in immunosurveillance [29]. Therefore, TIM-3 could become a promising target for cancer immunotherapy [30]. CD3⁺ T cells with increased expression of both inhibitory molecules, PD-1 and TIM-3, found in this study in patients with worse prognosis in stage IIB and IIC, could exhibit severe exhausted function as seen by their failure in IFN- γ production. This suggests that these two

molecules and their pathways work synergistically in establishing T-cell inhibition [31]. Therefore, the therapeutic blockade of both TIM-3 and PD-1 pathways might be very effective in controlling tumor growth and could restore anti-tumor immunity in cutaneous melanoma patients especially in stage IIB and IIC [32,33]. However, there was no difference in the expression of all analyzed inhibitory molecules, PD-1, TIM-3, and LAG-3 on CD3⁺ T cells in SLNs of melanoma patients in stage IIB, IIC, as well as in stage III. Yaddanapudi et al. [24] have shown that the patients with melanoma-positive SLNs had increased PD-1 expression on T cells. As we have only six patients in stage III with melanoma-positive SLNs in this research, additional investigations are necessary. Based on the results of this study, we could not determine exactly which patients in stage IIB and IIC would be the best candidates for the treatment with immune checkpoint inhibitors. In future research, more patients are needed for this analysis although FDA now provides this therapy to all patients in stage IIB and IIC for 12 months [10].

In physiological conditions, Treg cells have the role to establish the tolerance to self-antigens, while in cancer patients, they suppress immune responses against tumor-associated antigens [34]. In this study, melanoma patients in stage IIB and IIC had significantly higher percentage of Foxp3⁺ and CD4⁺ Foxp3⁺ Treg cells in SLNs compared with the percentage of these cells in patients in stage I and IIA. The presence of high number of FoxP3⁺ Tregs in SLNs of these melanoma patients can indicate an immunosuppressive TME, which is a known factor in promoting cancer progression and can be associated with the development of metastasis in cutaneous melanoma patients with worse prognosis [35]. Gupta et al. [36] have shown a significant association between the number of FoxP3⁺ cells in SLNs and the size of primary breast invasive ductal carcinoma. Furthermore, Viguier et al. [37] have found that CD4⁺CD25^{high} Treg cells were overrepresented in metastatic lymph nodes compared with both tumor-free LNs and autologous peripheral blood mononuclear cells. Tregs by the production of immunosuppressive cytokines, TGF- β , IL-10, and IL-35 or directly by cell-to-cell contact could decrease the percentage of total CD3⁺ and CD3⁺CD8⁺ CTLs, IFN- γ expression in CD3⁺ T cells, and could increase the expression of inhibitory PD-1 and TIM-3 molecules [38,39] that are found in stage IIB and IIC melanoma patients in this study. Also, these cells express high levels of enzymes, CD39 and CD73, that have the role to convert extracellular ATP into adenosine. The extracellular adenosine binds to A2A and A2B receptors on the surface of effector T cells and

impairs their proliferation, cytokines production, and cytotoxic anti-tumor activities [40]. All of these immunosuppressive mechanisms allow tumor cells to escape immune surveillance but also contribute to resistance to cancer immunotherapy.

In this study, we found the negative correlation between primary tumor depth in millimeters (Breslow) and the percentage of total CD3⁺, as well as CD3⁺CD8⁺ T cells in SLNs of all investigated melanoma patients. Furthermore, a positive correlation between Breslow thickness and the percentage of CD3⁺PD-1⁺ T lymphocytes was found. This result is in accord with the results of Yaddanapudi et al. [24] of positive correlation between Breslow thickness and frequency of immunosuppressive CD8⁺CD57⁺PD-1⁺ cells in SLNs of melanoma patients in stages I, II, and III. In addition to tumor thickness, ulceration of primary melanoma represents a critical, independent poor prognostic factor [10]. It has been shown that ulcerated melanoma had high density of macrophages and DCs that together with tumor cells produce immunosuppressive IL-6 [41]. High IL-6 could be associated with decreased percentage of antitumor CD3⁺ and CD3⁺CD8⁺ T cells, as well as increased percentage of CD3⁺PD-1⁺ cells found in this study for melanoma patients with ulcerated tumors. These results confirm once more that patients with thicker and ulcerated melanoma with worse prognosis have lower percentage of antitumor cells, as well as increased percentage of functional exhausted CD3⁺ T cells with high PD-1 expression in their SLNs. Furthermore, it is known that the presence of tumor-infiltrating lymphocytes (TILs) in primary melanoma is protective factor for lymph node metastasis [42,43]. However, in our study, we did not find any difference in the percentage of T-cell subsets and the expression of inhibitory checkpoint molecules, PD-1, TIM-3, and LAG-3, on CD3⁺ T cells between melanoma patients with focal (non-brisk) and dense (brisk) infiltrate in primary tumors. As we do not have the data in medical documentation for 10 melanoma patients regarding lymphocyte infiltration, additional investigations are needed.

Cutaneous melanoma is a heterogeneous cancer that consists of various histologic subtypes among which the most common are SSM (70% of the patients) and NM (20%) [44]. It is important to determine the histologic subtype of melanoma, as it has a prognostic significance for patients. It is known that the tumors of patients with NM have a higher Breslow thickness, more frequent ulcerations, higher dermal mitotic rate, and more frequent satellite lesions [45]. Therefore, NM patients have a more aggressive clinical course of disease with higher rates of recurrence and me-

tastasis compared with patients with SSM subtype [46,47]. Furthermore, molecular analyses show the differences between SSM and NM subtypes. It is known that NM patients contain a lower mutational load compared with SSM [48], as well as decreased presence of TILs [49] and an upregulation of PD-L1 expression on tumor cells [50]. In this study, we have shown for the first time statistically significant decrease in the percentage of cytotoxic CD3⁺CD8⁺ T cells in SLNs of patients with NM compared with SSM patients. Furthermore, we have found that the patients with NM had a trend of increased expression of PD-1, TIM-3, as well as LAG-3 inhibitory molecules on their CD3⁺ cells. These data suggest a weaker response and evasion of immune system in SLN of NM patients compared with SSM. These differences in immune characteristics of melanoma histologic subtypes contribute to more aggressive nature of NM subtype. It is known that immunotherapy is more effective in melanoma subtypes with a higher mutation rate and TIL presence [51]. However, it is unclear whether the patients with NM have a lower efficiency of immunotherapy compared with SSM. Lattanzi et al. [52] observed no difference in survival outcomes of NM versus SSM patients treated with immunotherapy (anti-PD-1, anti-CTLA-4 mAbs), while Pala et al. [53] displayed an improved survival of NM patients treated with immunotherapy compared with SSM patients. However, we would probably find more statistically significant differences in the analyzed parameters between SSM and NM patients if there were not even 14 patients for whom we do not have data regarding the histopathological type of their primary tumors in medical documentation. For that reason, we did not include these 14 patients and one patient with acral lentiginous melanoma in the statistical analysis. Furthermore, there are no data in the literature regarding the association between the functional and immunophenotypical characteristics of T cells in SLNs and different localizations of primary melanoma. In this study, we have found for the first time increased expression of inhibitory checkpoint molecules, especially LAG-3, on CD3⁺ T cells in non-malignant SLNs of melanoma patients with localization of tumor on extremities. According to these results, TME in SLNs of these patients could be immunosuppressive. On the other hand, no difference was found in the percentage of T cell subsets between patients with localization of melanoma on extremities and trunk. Fadaki et al. [54] showed a statistically significant SLN-positive rate for melanoma patients with trunk localization compared with localization on extremities. However, more research is needed to fully understand the distinct biological mechanisms and potential therapeutic differences between different melanoma histological subtypes and localizations.

Our results have shown a lower percentage of total CD3⁺ and CD3⁺CD8⁺ T lymphocytes, some lower IFN- γ expression in CD3⁺ T cells, as well as their higher expression of inhibitory PD-1 and TIM-3 molecules in melanoma patients with thicker ulcerated melanoma and worse prognosis in stage IIB and IIC. This functional exhaustion of T lymphocytes could be associated with a higher percentage of immunosuppressive Treg cells found in this research in these patients. These immunological alterations could cause progressive immune dysfunction in SLNs before the appearance of tumor cells in them. The mechanisms of these changes are very complex and multifactorial and further investigations are necessary to elucidate them in more detail. Therefore, immunosuppressive soluble mediators, cells, and signaling pathways in PMN of SLN might be targeted to inhibit the formation of PMN, repair antitumor immune response and prevent the occurrence of metastasis in SLN. This study provides the new results of the impairment of immune response in SLN of high-risk cutaneous melanoma patients, prior to lymphogenous metastasis and could help in understanding the need to introduce the new immunotherapies that could revive immune response and prevent melanoma metastasis in SLN. However, as the sample size of different clinical stages of melanoma patients analyzed in this study is relatively small, additional investigations and more patients are needed to improve this research.

Ethics Statement

All procedures performed in this study were approved by the Ethics Committee of Institute of Oncology and Radiology of Serbia (No. 14/2024/1081, date: April 3, 2024) in accordance with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained from all individual participants included in the study.

Availability of Data and Material

All data generated or analyzed during the study are included in this published article. Any further information related to this study can be obtained by contacting the authors.

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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Hyalinizing trabecular tumors with areas resembling noninvasive follicular thyroid neoplasm with papillary-like nuclear features: an immunohistochemical and molecular analysis

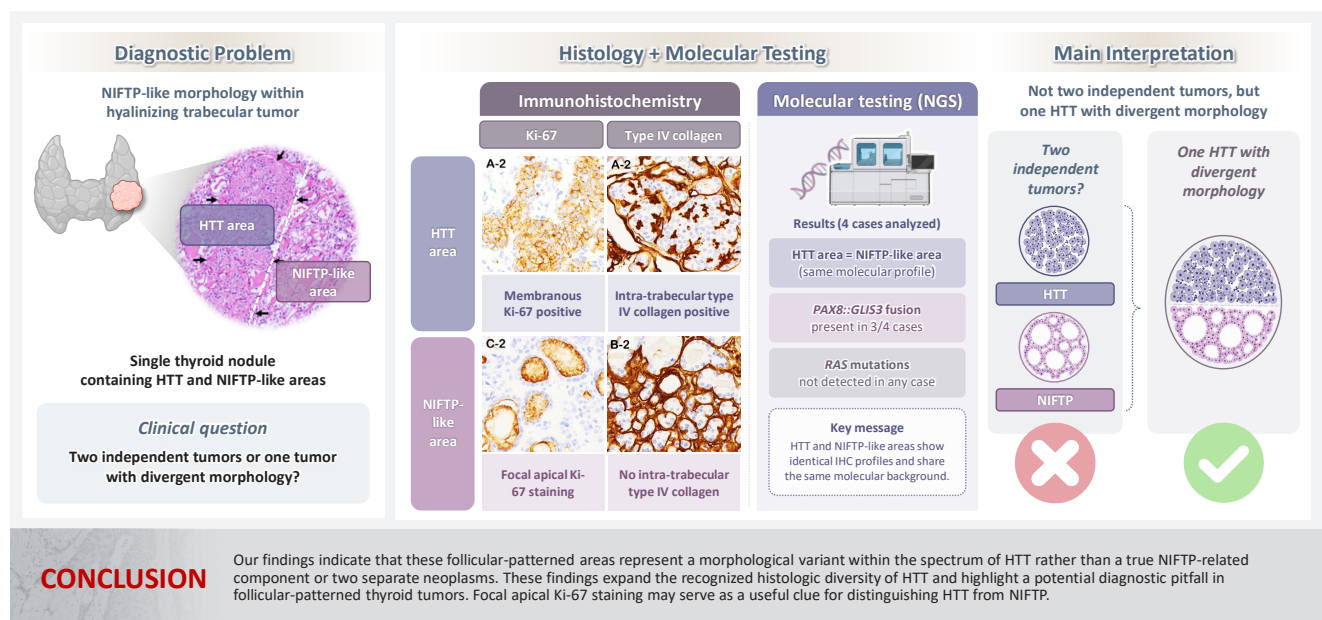
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Graphical abstract



Hyalinizing trabecular tumors with areas resembling noninvasive follicular thyroid neoplasm with papillary-like nuclear features: an immunohistochemical and molecular analysis

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Background: The coexistence of hyalinizing trabecular tumor (HTT) and areas with a morphology of noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) within a single thyroid nodule has not been previously reported. We aimed to determine whether such tumors represent two independent neoplasms or a single tumor exhibiting divergent morphology. **Methods:** Ten tumors containing both HTT and NIFTP-like areas were examined. The term “NIFTP-like” was used strictly as a descriptive morphological designation for areas that fulfill the histologic criteria of NIFTP. Immunohistochemical analyses of Ki-67 (MIB-1) and type IV collagen and targeted molecular testing were performed. Ten NIFTPs, 10 follicular adenomas, and three HTTs were used as controls. **Results:** HTT components consistently showed characteristic membranous Ki-67 staining and intra-trabecular type IV collagen deposition, whereas NIFTP-like areas lacked these features, except for focal apical Ki-67 staining. Intranuclear cytoplasmic inclusions in HTT were positive for type IV collagen. NIFTPs showed neither membranous Ki-67 nor intra-trabecular type IV collagen. Molecular analysis demonstrated identical profiles between HTT components and NIFTP-like areas: three tumors harbored *PAX8::GLIS3* fusions, and none showed *RAS* mutations. Pure HTT controls exhibited the same pattern. **Conclusions:** Our findings indicate that these follicular-patterned areas represent a morphological variant within the spectrum of HTT rather than a true NIFTP-related component or two separate neoplasms. These findings expand the recognized histologic diversity of HTT and highlight a potential diagnostic pitfall in follicular-patterned thyroid tumors. Focal apical Ki-67 staining may serve as a useful clue for distinguishing HTT from NIFTP.

Keywords: Thyroid neoplasms; Gene fusion; Type IV collagen; *RAS* mutation; Mutation; Immunohistochemistry

INTRODUCTION

Hyalinizing trabecular tumors (HTT) are uncommon thyroid neoplasms characterized by a trabecular growth pattern, prominent inter- and intra-trabecular hyaline material, and distinctive membranous immunoreactivity for MIB-1 (a Ki-67 clone) [1,2]. While inter-trabecular hyaline material is usually evident on routine hematoxylin and eosin staining, intra-trabecular

hyalinization can be subtle in some cases. Type IV collagen immunostaining is useful in such situations as it highlights basement-membrane-derived hyaline material and facilitates the recognition of HTT components [3]. HTT is currently classified as a low-risk thyroid tumor [1]. Cytologically, HTT tumor cells exhibit nuclear grooves, intranuclear cytoplasmic inclusions, and nuclear clearing, features that overlap with papillary thyroid carcinoma (PTC), making accurate diagnosis

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challenging. The recent identification of the *PAX8::GLIS3* fusion as a highly specific molecular hallmark has substantially improved the distinction of HTT from other follicle-derived thyroid tumors [3-6]. Noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) is also categorized as a low-risk tumor and is defined by a follicular growth pattern and PTC-like nuclear features [1]. Previously classified as a noninvasive encapsulated follicular variant of PTC [7], NIFTP is typically associated with *RAS*-like mutations, which are not specific and may also be observed in other benign or low-grade neoplasms [8,9]. Thus, although HTT and NIFTP share overlapping nuclear morphology and similarly indolent biological behavior, their growth patterns and molecular pathogenesis are fundamentally different.

During routine diagnostic practice, we identified a series of thyroid tumors demonstrating the coexistence of HTT and areas with NIFTP morphology within a single nodule. To our knowledge, such tumors have not been previously described. These lesions raise important questions regarding their histogenesis: they may represent a hybrid tumor composed of distinct HTT and NIFTP, or a single neoplasm exhibiting divergent differentiation. Therefore, the objective of this study was to determine whether the coexistence of HTT and areas with NIFTP morphology within a single nodule represents two independent neoplasms or a single tumor with divergent morphology. To address this question, we integrated detailed histological assessment with immunohistochemical and molecular analyses, with particular emphasis on detecting *PAX8::GLIS3* fusion. Our goal was to clarify the histogenesis of these unusual tumors and to identify diagnostic features that may help to avoid misclassification in routine practice.

MATERIALS AND METHODS

Case selection

We retrospectively reviewed data from 70 HTTs that were resected at our institution between 1983 and 2024. Among these, we identified 10 tumors demonstrating the coexistence of HTT components and NIFTP-like areas, defined as the presence of both components within the same nodule, with the NIFTP-like areas comprising at least 1% of the total tumor area (Fig. 1). The HTT components were defined as comprising intra-trabecular hyalinized material and having a trabecular growth pattern and PTC-type nuclear features, including nuclear grooves, pseudoinclusions, and membrane irregularities. The

NIFTP-like area was defined as having a follicular growth pattern without intratrabecular hyalinized material, and showing the existence of PTC-like nuclear features (nuclear score of 2 or 3), an absence of tumor necrosis, and a low mitotic count (<3 mitosis/ 2 mm^2). The term “NIFTP-like” was used strictly as a descriptive morphological designation for areas that fulfill the histologic criteria of NIFTP on hematoxylin and eosin (H&E) sections. This terminology does not imply true NIFTP; rather, it was applied because the follicular-patterned morphology is not inconsistent with the histologic features of NIFTP. All histopathological evaluations and case selections were independently reviewed and confirmed by two board-certified pathologists specializing in thyroid pathology (Mitsuyoshi Hirokawa and Hiroshi Kamma). Both pathologists examined the slides in a single-blind manner with respect to the molecular results, and discrepancies were resolved by joint review and consensus. For immunohistochemical and molecular analyses, we selected as controls 10 classic and pure NIFTP, 10 follicular adenoma, and three HTT cases from among the most recent cases within the study period. Formalin-fixed paraffin-embedded (FFPE) tissue blocks from all selected cases were retrieved for histological, immunohistochemical, and molecular analyses.

Immunohistochemistry

Immunohistochemical staining was performed using antibodies against Ki-67 (MIB-1, 1:200, Dako Cytomation, Carpinteria,

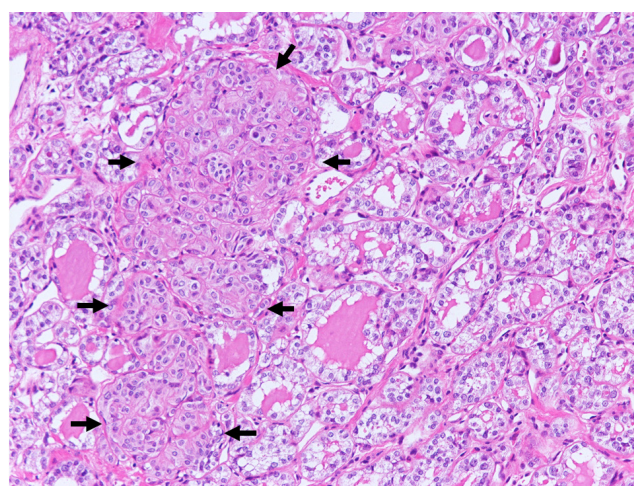


Fig. 1. Typical HTT components (arrows) and NIFTP-like areas within a single nodule. HTT, hyalinizing trabecular tumor; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

CA, USA) and type IV collagen (PHM-12, 1:100, Merck KGaA, Darmstadt, Germany). To evaluate characteristic membranous reactivity, Ki-67 immunostaining was specifically selected for assessing the HTT component. Type IV collagen immunostaining was included to facilitate the identification of intra-trabecular hyalinization, which can be difficult to recognize on routine hematoxylin and eosin staining. Staining was carried out on an automated Leica Bond-Max system using the Bond Refine detection kit (Leica Microsystems, Bannockburn, IL, USA). For Ki-67 immunohistochemistry, antigen retrieval was performed manually using heat treatment (electric kettle 98°C, 40 minutes) [10].

Molecular analysis

As the study cohort spanned more than 40 years, many archival specimens were older than 5 years, which is considered unsuitable for reliable next-generation sequencing (NGS) analysis. In addition, separate macrodissection of the HTT components and NIFTP-like areas required each area to contain at least a 5-mm square of viable tissue. Only four tumors met both criteria. These correspond to cases 7, 8, 9, and 10 in Table 1, and the same case numbers are used in Table 2 to indicate the tumors that underwent molecular testing. FFPE tissue fragments were separately microdissected from HTT components and NIFTP-like areas. DNA and RNA were extracted using the AllPrep DNA/RNA FFPE Kit (#80234, Qiagen, Hilden, Germany). Molecular analysis was performed using a targeted Japanese NGS panel (ThyroGene v3, Nasu Medical Institute of Medical Sciences, Tochigi, Japan), which detects the most common genetic alterations in thyroid tumors, including *PAX8::GLIS1/3* fusions, *BRAFV600E* mutations, and *RAS* (*KRAS*, *HRAS*, and *NRAS*) mutations.

RESULTS

A total of 10 tumors containing both HTT components and NIFTP-like areas were identified among 70 resected HTTs (14.3%). The clinical and pathological characteristics of these tumors are summarized in Table 1. Patients ranged from 28 to 75 years of age, and eight were women. Tumor sizes varied from 6 to 54 mm. All cases had been originally diagnosed as HTT. Encapsulation was absent or partial in all tumors. HTT components predominated in seven cases, whereas NIFTP-like areas predominated in three cases. Nuclear scores were uniformly high (score 3 in both HTT components and NIFTP-like areas).

HTT components were distributed in a geographic or is-

land-like pattern in all cases, and their proportions varied considerably across tumors. In seven of the 10 cases, HTT components accounted for >50% of the tumor area (Fig. 2A), whereas in the remaining three cases, NIFTP-like areas predominated (>50%) (Fig. 2B). The two components were intimately intermingled, and their boundaries could not be clearly delineated.

HTT components consistently exhibited characteristic membranous immunoreactivity for Ki-67 (Fig. 3A, D). In contrast, most areas of the NIFTP-like areas lacked Ki-67 staining (Fig. 3B, E). However, focal apical Ki-67 immunostaining was observed, predominantly in areas adjacent to HTT components (Figs. 3C, F, 4).

All HTT components showed intratrabecular deposition of type IV collagen-positive material (Fig. 5A, D), whereas this feature was absent in NIFTP-like areas (Fig. 5B, E). In addition, the intranuclear cytoplasmic inclusions in the HTT components were positive for type IV collagen (Fig. 5C, F).

None of the control cases (pure NIFTPs, follicular adenomas, or normal thyroid follicular cells) showed membranous or apical Ki-67 immunoreactivity. We observed type IV collagen deposition surrounding tumor nests but not between tumor cells.

Genetic analyses of four tumors containing both HTT components and NIFTP-like areas demonstrated that the two components shared identical molecular profiles (Table 2). In three tumors (cases 7–9), both components harbored *PAX8::GLIS3* fusion, whereas no *RAS* (*KRAS*, *HRAS*, and *NRAS*) mutations, characteristic of NIFTP, were detected. The remaining tumor showed no *PAX8::GLIS3* fusion or *RAS* mutations. No additional point mutations or gene fusions, including *BRAFV600E*, *RET*, or *PAX8::PPAR γ* rearrangements, were identified in any tumors. No cases showed *TERT* promoter hotspot mutations (C228T/C250T), although the *rs2853669* polymorphism was present with variable genotypes.

Three pure HTT tumors were included as controls; all demonstrated *PAX8::GLIS3* fusion and lacked *RAS* mutations. *TERT* promoter hotspot mutations were absent, and *rs2853669* was detected in all cases.

DISCUSSION

All ten tumors examined in this study contained areas fulfilling established diagnostic criteria for HTT [1,2,11]. These areas consistently exhibited a trabecular growth pattern with PTC-like nuclear features, prominent intratrabecular hyalinization, and characteristic membranous Ki-67 immunoreactivity. In ad-

Table 1. Clinical and pathological characteristics of 10 tumors with HTT and NIFTP-like areas

Case No.	Age (yr)	Sex	Tumor size ^a (mm)	Location	Original diagnosis	Encapsulation	Predominant histology	Nuclear score		Ki-67 membranous staining		Ki-67 apical (NIFTP-like)	Type IV collagen (intra-trabecular)		Type IV collagen-positive pseudoinclusions (HTT)
								HTT	NIFTP-like	HTT	NIFTP-like		HTT	NIFTP-like	
1	62	F	30	Left lobe	HTT	Partial	HTT	3	3	+	-	+	+	-	+
2	28	M	54	Right lobe	HTT	Partial	NIFTP	3	2	+	-	+	+	-	+
3	64	F	6	Isthmus	HTT	Partial	HTT	3	3	+	-	+	+	-	+
4	66	M	40	Left lobe	HTT	Absent	HTT	3	3	+	-	+	+	-	+
5	75	F	17	Right lobe	HTT	Absent	HTT	3	3	+	-	+	+	-	+
6	42	F	16	Right lobe	HTT	Absent	HTT	3	3	+	-	+	+	-	+
7	45	F	15	Isthmus	HTT	Partial	HTT	3	3	+	-	+	+	-	+
8	56	F	23	Right lobe	HTT	Partial	HTT	3	3	+	-	+	+	-	+
9	35	F	15	Right lobe	HTT	Absent	NIFTP	3	3	+	-	+	+	-	+
10	55	F	11	Right lobe	HTT	Absent	NIFTP	3	3	+	-	+	+	-	+

HTT, hyalinizing trabecular tumor; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; +, presence; -, absence.

^aGreatest dimension.

Table 2. Molecular profiles of the four tumors (cases 7–10) that met eligibility criteria for NGS analysis

Case	Histological component	Point mutations				Fusions				TERT promoter	
		RAS(K/H/M)/RAS-like	BRAF (V600E)	RET	Others	PAX8::PPARG	RET::CCDC6	PAX8::GLIS3	PAX8::GLIS1	C228T/C250T	rs2853669
7	HTT	-	-	-	-	-	-	-	-	-	T/T
	NIFTP	-	-	-	-	-	-	-	-	-	-
8	HTT	-	-	-	-	-	-	-	-	-	C/T
	NIFTP	-	-	-	-	-	-	-	-	-	-
9	HTT	-	-	-	-	-	-	-	-	-	C/T
	NIFTP	-	-	-	-	-	-	-	-	-	-
10	HTT	-	-	-	-	-	-	-	-	-	T/T
	NIFTP	-	-	-	-	-	-	-	-	-	-
Control 1	HTT	-	-	-	-	-	-	-	-	-	C/T
Control 2	HTT	-	-	-	-	-	-	-	-	-	C/T
Control 3	HTT	-	-	-	-	-	-	-	-	-	C/C

Only four tumors (cases 7–10 in Table 1) fulfilled the technical requirements for molecular testing, including (1) archival tissue age ≤5 years to ensure reliable NGS performance and (2) availability of ≥5-mm viable tissue in both HTT and NIFTP-like areas for separate macrodissection. The same case numbers are used in Table 2 to indicate the tumors that underwent molecular analysis.

NGS, next-generation sequencing; HTT, hyalinizing trabecular tumor; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; +, present; -, absent.

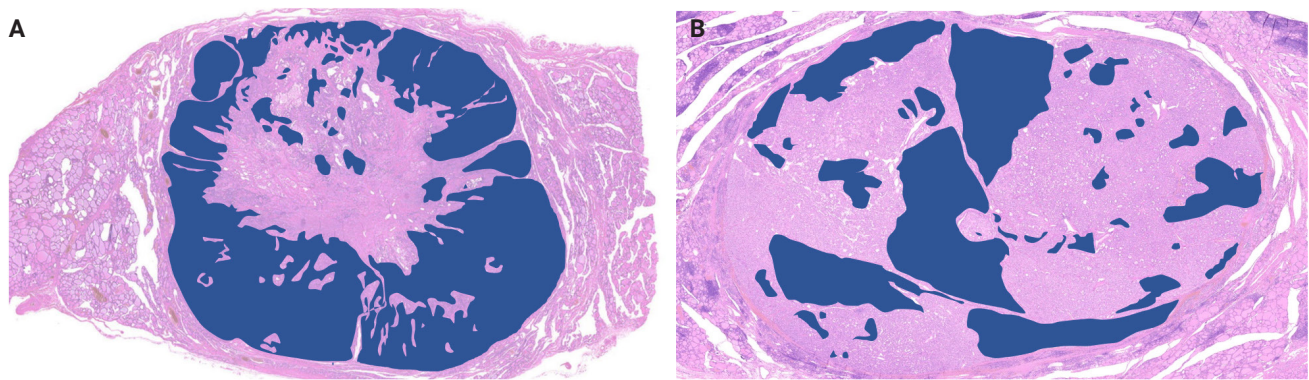


Fig. 2. Distributions of HTT components (dark blue) and NIFTP-like areas. (A) Predominantly HTT components. (B) Predominantly NIFTP-like areas. HTT, hyalinizing trabecular tumor; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

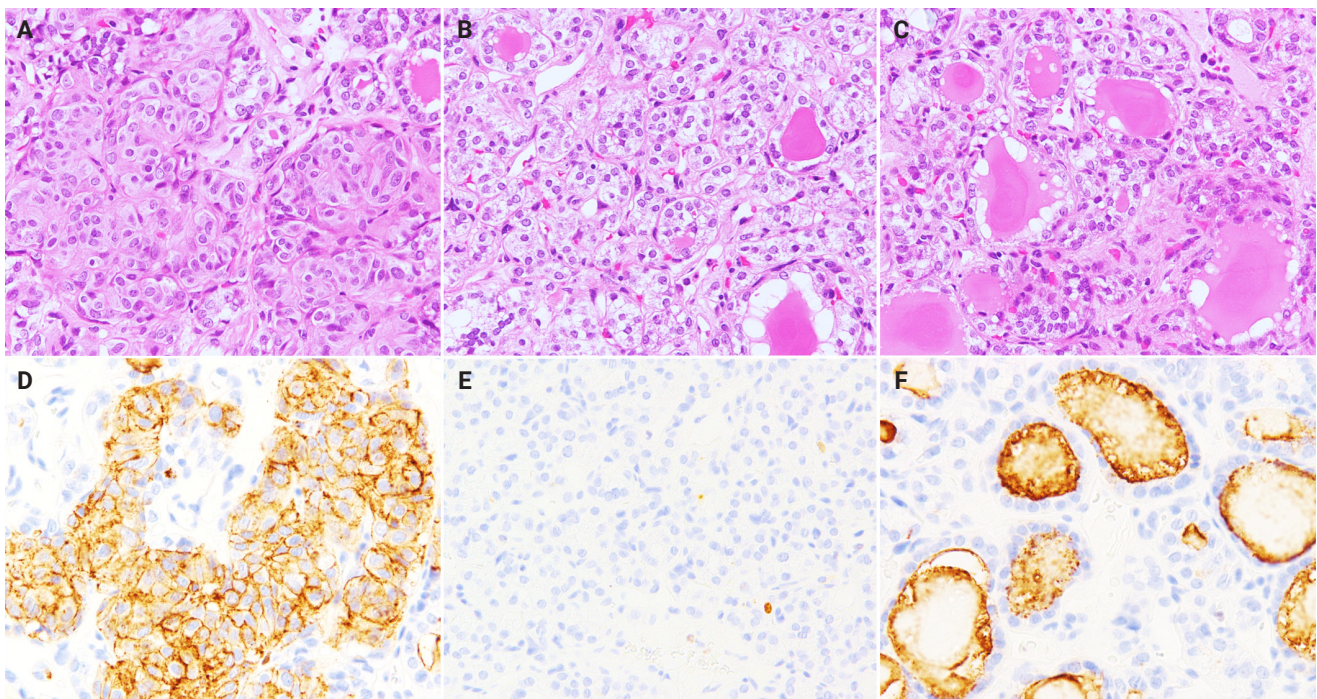


Fig. 3. Ki-67 immunohistochemical staining and corresponding H&E morphology in HTT and NIFTP-like areas. (A) Typical HTT component showing a trabecular growth pattern with intra-trabecular hyalinization. (B) NIFTP-like area exhibiting a follicular growth pattern without intra-trabecular hyalinization. (C) NIFTP-like area adjacent to HTT components. (D) Corresponding Ki-67 immunostaining demonstrating characteristic membranous reactivity in HTT cells. (E) Corresponding Ki-67 immunostaining showing absence of membranous staining in the NIFTP-like area. (F) Corresponding Ki-67 immunostaining demonstrating apical reactivity in areas close to HTT components. H&E, hematoxylin and eosin; HTT, hyalinizing trabecular tumor; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

dition, NGS confirmed the presence of *PAX8::GLIS3* fusion in three of four cases tested, which, together with the histological and immunophenotypic findings, is highly specific and essentially pathognomonic for HTT [3-6]. Therefore, a diagnosis of HTT based on these components is unequivocal.

In contrast, other areas within the same tumors showed a predominantly follicular growth pattern with PTC-type nuclear features but lacked papillary architecture, the defining features of HTT, and evidence of invasive growth. These findings are not inconsistent with the histological diagnostic criteria for

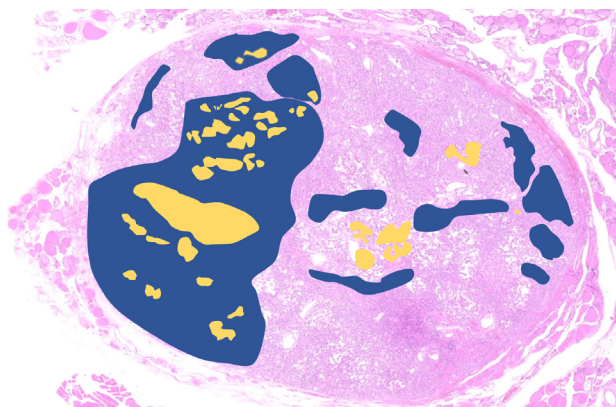


Fig. 4. Distribution of Ki-67 immunoreactivity in HTT and NIFTP-like areas. Dark blue areas: HTT components with characteristic membranous Ki-67 immunoreactivity; yellow areas: NIFTP-like areas showing apical Ki-67 staining; remaining areas: NIFTP-like areas lacking Ki-67 immunoreactivity. HTT, hyalinizing trabecular tumor; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

NIFTP [7]. Accordingly, each tumor appeared to contain two morphologically distinct components: one consistent with the HTT component and the other resembling NIFTP, coexisting within a single lesion.

To further elucidate the nature of these unusual lesions, we performed molecular analyses focusing on *PAX8::GLIS3* fusion and *RAS* mutations. Both HTT components and NIFTP-like areas shared identical molecular profiles: *PAX8::GLIS3* fusion was detected in three of four cases, and no *RAS* mutations were identified in any tumors. This molecular concordance strongly argues against the presence of two independent neoplasms. Instead, the findings support the interpretation that the NIFTP-like areas represent a morphological variant within the spectrum of HTT rather than a distinct coexisting tumor.

Our results demonstrate that HTT can exhibit areas closely resembling NIFTP morphology, a phenomenon not previously described to our knowledge. This observation expands the recognized morphological spectrum of HTT and highlights

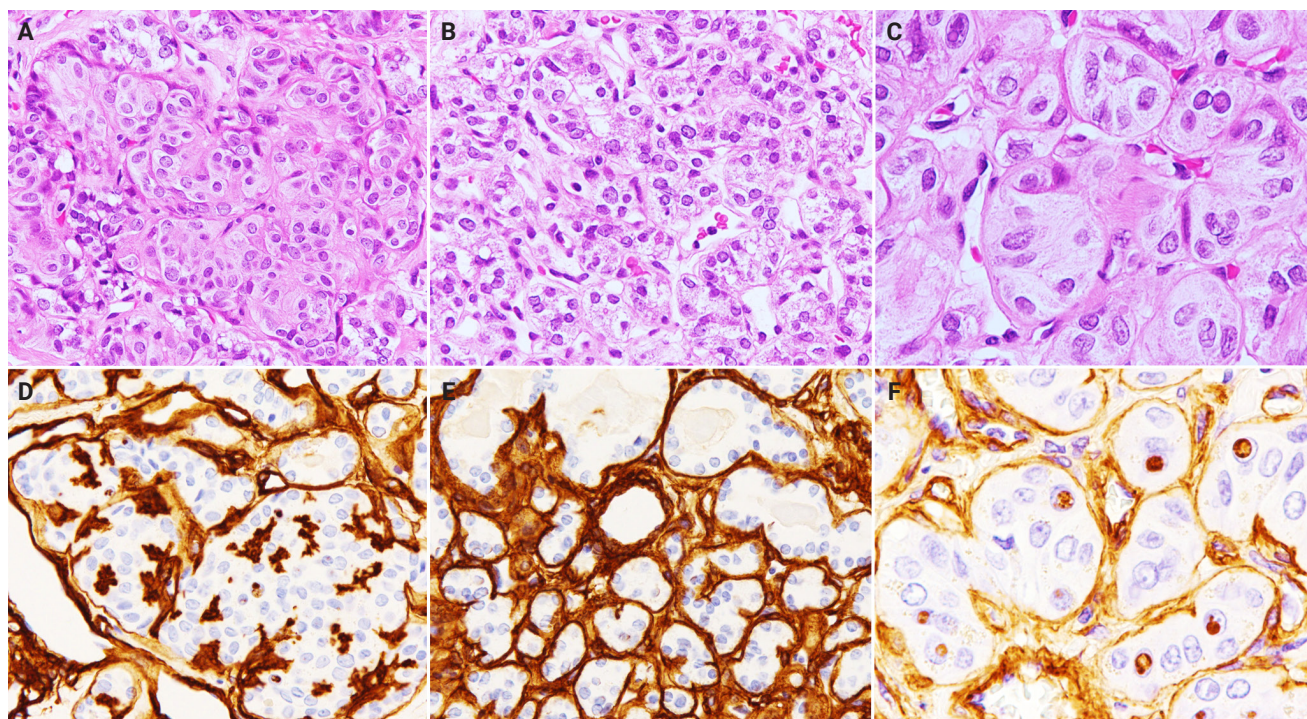


Fig. 5. Type IV collagen immunoreactivity and corresponding H&E morphology in HTT and NIFTP-like areas. (A) Typical HTT components showing a trabecular growth pattern with prominent intratrabeal hyalinization. (B) NIFTP-like area exhibiting a follicular growth pattern without intra-trabeal hyalinization. (C) HTT components containing intranuclear cytoplasmic inclusions on H&E staining. (D) Corresponding type IV collagen immunostaining demonstrating deposition in both inter- and intra-trabeal areas. (E) Corresponding type IV collagen immunostaining showing absence of intra-trabeal deposition. (F) Corresponding type IV collagen immunostaining demonstrating positivity within the intranuclear cytoplasmic inclusions. H&E, hematoxylin and eosin; HTT, hyalinizing trabecular tumor; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

its greater heterogeneity than that traditionally appreciated. In tumors with a predominant NIFTP-like pattern, the characteristic features of HTT may be subtle or overlooked, creating a potential diagnostic pitfall. In such settings, focal apical Ki-67 immunoreactivity may be a useful ancillary clue supporting the diagnosis of HTT. Accordingly, HTT should be considered in the differential diagnosis of follicular-patterned lesions with PTC-type nuclear features to avoid misclassification as NIFTP.

An additional notable finding was the presence of type IV collagen with intranuclear cytoplasmic inclusions in HTT cells. These inclusions are generally regarded as cytoplasmic invaginations surrounded by the nuclear membrane [12-14] and, in PTC, are not known to contain basement membrane components [13]. Because type IV collagen is a basement membrane protein [14,15], its presence within these inclusions was unexpected and should theoretically not occur if the inclusions consisted solely of cytoplasmic material. One possible explanation is that the intranuclear cytoplasmic inclusions in HTT incorporate basement membrane substances, including type IV collagen. Regardless of the underlying mechanism, this feature may serve as a useful diagnostic clue for distinguishing HTT from PTC.

This study has several limitations. The sample size was small, reflecting the rarity of HTT and tumors with mixed HTT and NIFTP-like morphology. As the cohort spans more than 40 years, many archival specimens were older than five years and unsuitable for reliable NGS analysis, and separate macrodissection required each component to contain at least a 5-mm square of viable tissue; therefore, only four tumors were eligible for molecular testing. Although the two components were intermingled in many areas, macrodissection was performed only on clearly demarcated areas to minimize cross-contamination, and the absence of discordant driver alterations argues against contamination as the cause of the uniform molecular profiles. Case 10 lacked detectable *PAX8::GLIS3* fusion; however, fusion-negative HTTs have been reported, and the diagnosis should not rely solely on molecular findings when characteristic histological and immunophenotypic features are present. Finally, our molecular testing targeted selected alterations, and broader genomic profiling may further clarify the relationships between these components. Further studies with larger cohorts are required to validate our observations.

In summary, our findings demonstrate that tumors containing both HTT and NIFTP-like areas share a uniform molecular profile, including the presence of *PAX8::GLIS3* fusion. In this study, “NIFTP-like” refers only to the morphological resem-

blance to NIFTP and does not imply a separate neoplastic process. Taken together, the molecular and immunohistochemical concordance indicates that these follicular-patterned areas represent a morphological variant within the spectrum of HTT. This study clarifies the histogenesis of these tumors and highlights key diagnostic clues—particularly apical Ki-67 staining—that may prevent misclassification as NIFTP in follicular-patterned thyroid lesions.

Ethics Statement

All procedures performed in the current study were approved by the Institutional Review Board (IRB) of Kuma Hospital on October 12, 2023 (approval No. 20231012-4) in accordance with the 1964 Helsinki declaration and its later amendments. All study participants provided informed consent.

Availability of Data and Material

Data supporting the findings of this study are available from the corresponding author upon request.

Code Availability

Not applicable.

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Conflicts of Interest

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Non-keratinizing immature squamous metaplasia in the pancreas mimicking malignancy: a diagnostic pitfall in cytology

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This report describes a challenging case in which atypical immature squamous metaplasia was misinterpreted as malignancy. A 69-year-old man presented with abdominal pain and loss of appetite. Imaging revealed mild pancreatic duct dilation, parenchymal enlargement, and increased fat attenuation in the transverse mesocolon. Endoscopic ultrasound revealed a hypoechoic lesion in the pancreatic body. The serum amylase level was markedly elevated (1,785 U/L), consistent with acute pancreatitis. Repeated pancreatic juice cytology examinations demonstrated atypical epithelial clusters, which raised concerns about possible pancreatic ductal adenocarcinoma. Therefore, distal pancreatectomy with splenectomy and transverse colon resection were performed. However, histopathological examination revealed only atypical immature squamous metaplasia. Retrospective review of the cytological specimens showed overlapping cell clusters with coarse chromatin, prominent nucleoli, nuclear pleomorphism, and peripheral dissociation in a neutrophilic background with focal hemorrhagic necrosis. Although rarely encountered, squamous metaplastic cells can appear in pancreatic cytology and represent a potential pitfall by mimicking adenocarcinoma.

Keywords: Pancreas; Squamous metaplasia; Cytology; Pancreatitis; Misdiagnosis

INTRODUCTION

The reported frequency of squamous metaplasia in the pancreas varies widely in the literature [1,2]. According to the Armed Forces Institute of Pathology, squamous metaplasia is found in up to 45% of pancreata [3]. It typically arises in the intercalated ducts and is characterized by relatively immature squamous epithelial cells occupying the pancreatic ducts [3]. Notably, non-keratinizing squamous metaplasia is observed more frequently and has been referred to as “multilayered metaplasia” [3], “immature squamous metaplasia” [2], or “transitional metaplasia” [4].

There have been reports of morphologically identifiable keratinizing squamous metaplasia in the field of cytology [1,5,6]. However, to the best of our knowledge, non-keratinizing im-

mature squamous metaplasia in the pancreas has not been discussed, likely because cytology may not identify immature squamous metaplastic cells that are non-keratinized and lack characteristic features. We have encountered a case in which cytology initially led to a diagnosis of malignancy, but subsequent examination of the resected pancreatic specimen revealed only atypical immature squamous metaplasia. This report presents our findings in this extremely rare case in which immature squamous metaplastic cells were identified by cytology.

CASE REPORT

A 69-year-old man visited our hospital with complaints of abdominal pain and loss of appetite. He had no significant med-

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*This case was presented at the 22nd Korea-Japan Joint Meeting for Diagnostic Cytopathology, held in Yeosu, South Korea, on Saturday, September 27, 2025.

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ical history. However, he had a history of consuming 180 mL of Japanese sake per day. Abdominal computed tomography revealed mild dilation of the main pancreatic duct at the pancreatic tail and mild enlargement of the pancreatic parenchyma in the same region (Fig. 1). Furthermore, increased density was observed in the mesenteric adipose tissue in the area of the transverse colon. These findings suggested pancreatitis and fat necrosis of the mesenteric adipose tissue supporting the transverse colon. A 16-mm hypoechoic lesion with irregular margins was observed in the pancreatic body on endoscopic ultrasound; fine-needle aspiration was not performed because of the pancreatitis. Pancreatic enzyme assays showed marked elevations of serum and pancreatic amylase, reaching peak levels of 1,785 U/L, which supported the diagnosis of pancreatitis. Serum tumor markers, including S-pancreas antigen-1 (SPAN-1), Duke pancreatic antigen-2 (DUPAN-2), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen, were all within normal ranges. Endoscopic pancreatic duct drainage and stenting was performed, followed by repeated pancreatic juice cytology examinations, which were submitted 15 times over an 11-day period. Atypical cells were detected in the final six specimens and were interpreted as atypical cells once, suspicious for malignancy three times, and positive for malignancy once. Although

imaging findings were not definitive for malignancy, repeated pancreatic juice cytology demonstrated atypical epithelial clusters with features concerning for adenocarcinoma, leading to surgical intervention. Distal pancreatectomy combined with splenectomy, resection of the transverse colon, and cholecystectomy were performed. The postoperative course was uneventful. Given the possibility of occult microcarcinoma, the patient was followed according to a surveillance protocol similar to that for pancreatic malignancy. Computed tomography and serum tumor marker assessments were performed every 6 months. Although the serum carcinoembryonic antigen level fluctuated between 5 ng/mL and 8 ng/mL, no other clinical, radiological, or laboratory evidence of malignancy was identified during 5 years of follow-up, and surveillance was subsequently discontinued.

Cytological findings

Alcohol-fixed slides were stained by the Papanicolaou method in the cytology laboratory. Numerous cell clusters with overlapping arrangements were observed against a background of diffuse neutrophilic infiltration (Fig. 2A, B). Focal hemorrhagic necrosis was present (Fig. 2A). The cell clusters contained large cells with coarse nuclear chromatin, and nucleoli were prominent and enlarged (Fig. 2C). Nuclear pleomorphism was evident, with irregular nuclear arrangement and cell dissociation observed at the periphery of the clusters (Fig. 2D–H). Taken together, these cytological features of architectural complexity, nuclear atypia, and a necrotic inflammatory background supported a diagnosis of pancreatic ductal adenocarcinoma.

Histopathological findings

Macroscopically, a portion of the cut surface of the pancreas showed necrosis as a result of pancreatitis. However, no distinct mass was identified (Fig. 3A). Histologically, mild fibrosis and dilated peripheral pancreatic ducts were observed, consistent with chronic pancreatitis. Focal necrosis of the surrounding adipose tissue and pancreatic parenchyma was also present, indicating superimposed acute pancreatitis (Fig. 3B). Furthermore, the concurrently resected transverse colon showed extensive mesenteric necrosis, consistent with the preoperative computed tomography findings and supportive of marked acute pancreatitis. The epithelium of the main pancreatic duct was extensively denuded, and squamous metaplasia was widely observed in both the main pancreatic duct and its branches (Fig. 3C, D).

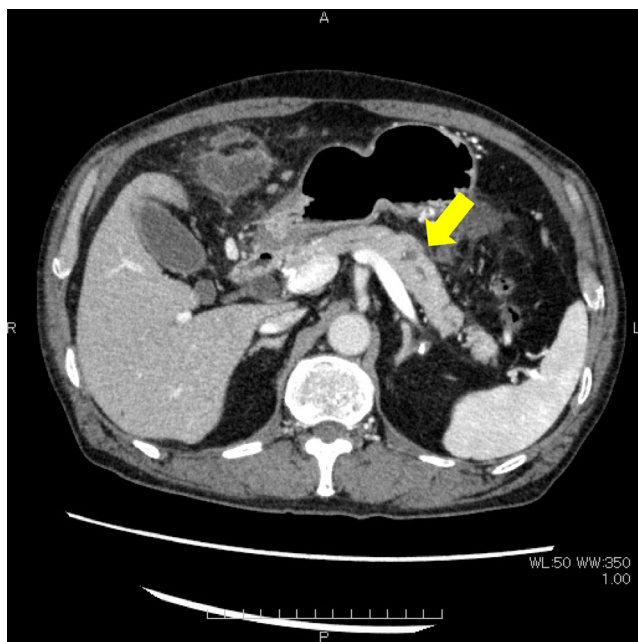


Fig. 1. Abdominal computed tomography scan showing mild dilatation of the main pancreatic duct at the pancreatic tail and mild enlargement of the pancreatic parenchyma in the same region (arrow).

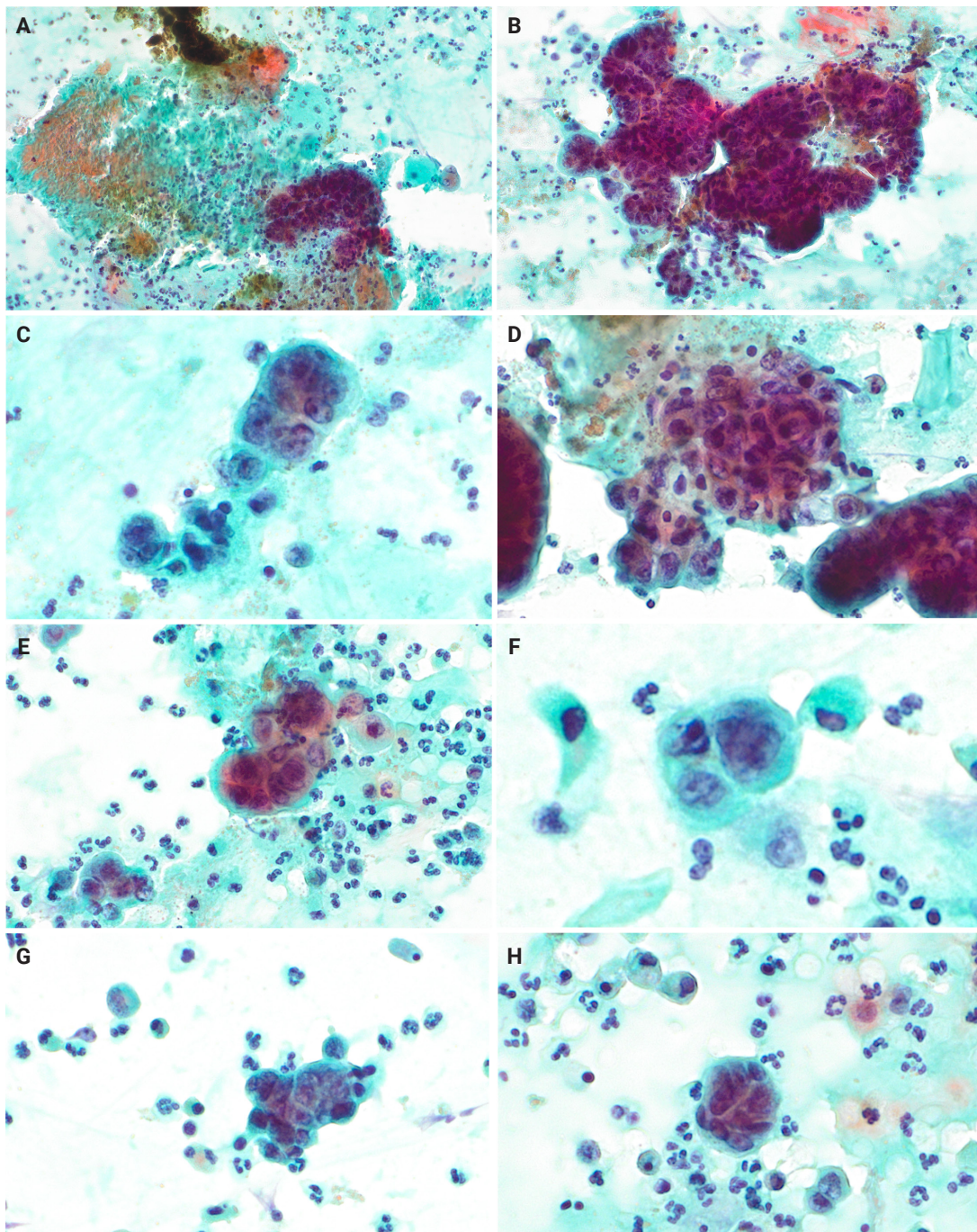


Fig. 2. Cytological findings. (A) Clusters of large epithelial cells within a necrotic background. (B) Large cell clusters with complex overlapping. (C–H) Cell clusters demonstrating pleomorphism with irregular nuclear contours, coarse chromatin, and prominent nucleoli. At the periphery, cell dissociation and scattered atypical cells are observed.

The squamous metaplastic cells varied in nuclear size, nuclear pleomorphism, and architectural disarray, closely resembling the atypical cells identified on cytological examination (Fig. 3E–G). Apart from the atypical immature squamous metapla-

sia, only a focal low-grade PanIN (pancreatic intraepithelial neoplasia)-equivalent mucinous epithelial metaplasia was observed in a single branch.

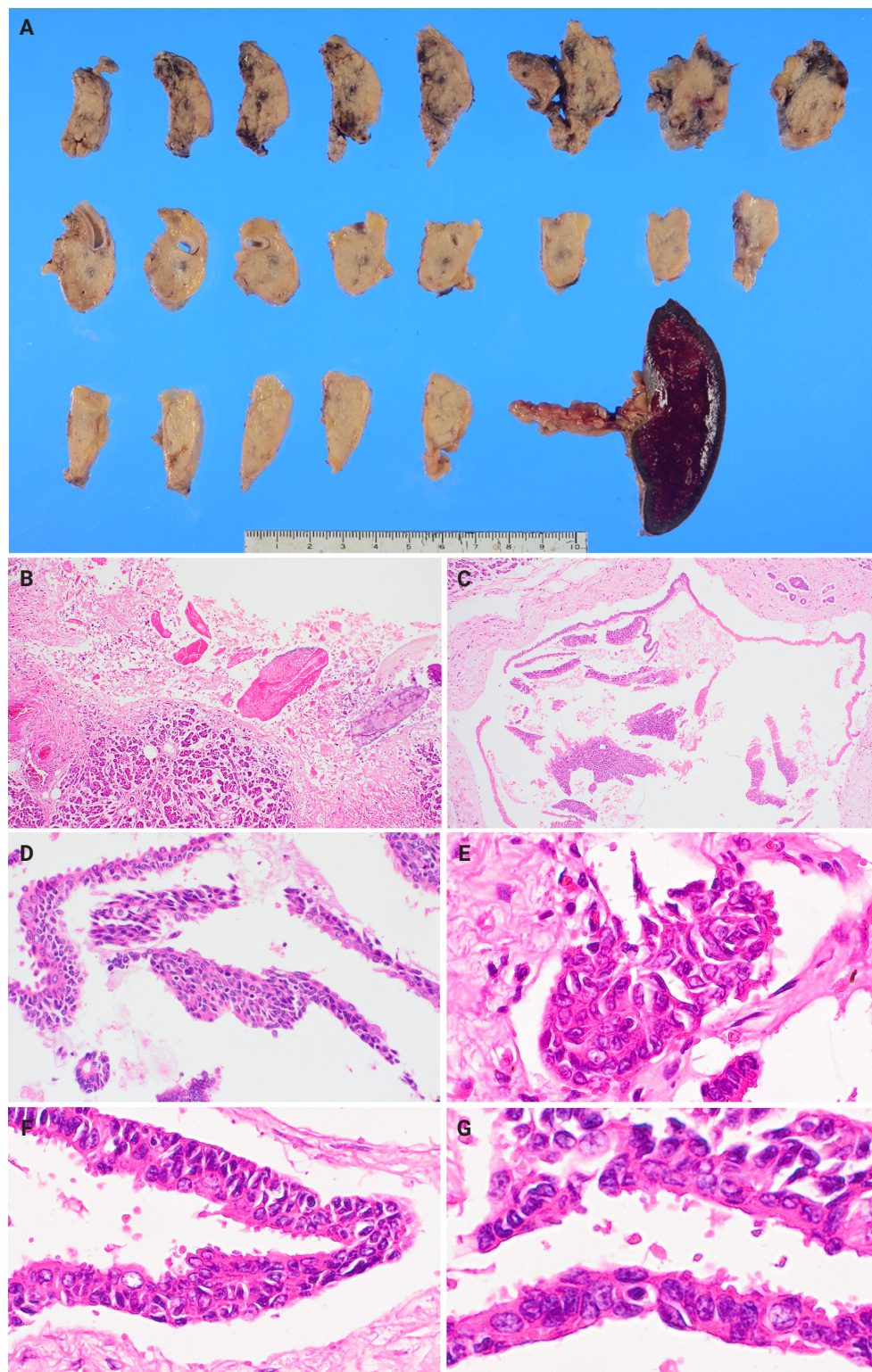


Fig. 3. Macroscopic and microscopic findings in the resected pancreas. (A) Macroscopic view of the cut surface of the resected pancreas. No obvious tumor was identified on gross examination. (B) Hemorrhagic necrosis in the main pancreatic duct. (C) Partial dilatation of the main pancreatic duct with foci of squamous metaplasia. (D–G) Higher magnification reveals nuclear atypia of the squamous epithelial cells, corresponding to the atypical epithelial cells observed on cytology.

DISCUSSION

The key diagnostic cytopathological features of pancreatic ductal adenocarcinoma include a necrotic background, loosely cohesive cell clusters, uneven nuclear spacing, loss of polarity (i.e., “drunken honeycomb”), hyperchromatic nuclei, and nuclear irregularity [7,8]. In the present case, the cytological specimens showed a necrotic and hemorrhagic background with clusters of varying sizes composed of large cells showing marked nuclear pleomorphism, irregular nuclear contours, coarse chromatin, and prominent nucleoli. Partial loss of cellular cohesion with cytologic features closely overlapped with those characteristic of pancreatic ductal adenocarcinoma led to an initial interpretation of malignancy. However, the hemorrhagic necrosis observed in the background was likely attributable to acute pancreatitis, based on clinical findings and the marked presence of neutrophils.

False-positive cytology results in patients with chronic pancreatitis have been reported previously [9-11]. Chronic pancreatitis causes reactive nuclear enlargement and pleomorphism of pancreatic ductal epithelium because of inflammation, which presents cytologically with features that can be mistaken for adenocarcinoma [11,12]. However, false positives caused by squamous metaplasia have not been discussed until now. In the present case, although findings suggestive of chronic pancreatitis were observed, they were limited to mild fibrosis, and the main pathology was acute pancreatitis.

Bixler et al. [1] described atypical squamous metaplasia as being characterized by mild to moderate nuclear hyperchromasia, nuclear enlargement up to four to five times the size of normal nuclei with pleomorphism, and nuclear irregularity with a relatively preserved nuclear-to-cytoplasmic ratio. Layfield et al. [6] reported that even when atypical squamous metaplastic cells are present, they can be distinguished from adenosquamous carcinoma or squamous cell carcinoma based on the number of atypical cells and the degree of nuclear atypia. Specifically, unlike squamous cell carcinoma or adenosquamous carcinoma, atypical squamous metaplastic cells tend to form cohesive clusters and lack necrotic debris in the background. However, in the present case, the atypical squamous metaplastic cells lacked overt keratinization and had scant cytoplasm with a high nuclear-to-cytoplasmic ratio, closely mimicking atypical glandular cells and leading to misinterpretation as adenocarcinoma.

Histologically, immature squamous metaplastic cells in the pancreas are readily identifiable in resected specimens, allowing

easy differentiation from malignancy, but can sometimes show atypia, necessitating differentiation from high-grade pancreatic intraepithelial neoplasia and other premalignant lesions [2,4]. In our case, atypical squamous metaplasia was identified histologically. The squamous metaplastic epithelial cells showed a tendency to detach from the ductal lining in both the main pancreatic duct and its branches. The morphology of these exfoliated cells closely resembled the atypical epithelial clusters observed in the cytological specimens, allowing retrospective recognition that the cytologically atypical cells corresponded to squamous metaplastic cells. On high-power examination, these cells were relatively large with architectural disarray, irregular nuclear contours, and coarse chromatin. Although these features represented marked cytological atypia at high magnification, low-power examination revealed unequivocal squamous metaplasia, and malignancy was not suspected histologically. These cells were considered to represent reactively enlarged squamous metaplastic cells with cytological atypia; however, when fragmented and exfoliated in pancreatic juice, they can closely mimic adenocarcinoma cells, as in the present case. This represents a significant diagnostic pitfall that has not been previously reported and should be carefully noted.

A retrospective review of the cytological findings in this case revealed several features that could have helped avoid this diagnostic error. Although it remains uncertain whether atypical squamous metaplasia could be reasonably suspected when a similar case is encountered in routine practice, several observations from this case may nonetheless provide limited but important clues. Although the cell clusters showed some degree of peripheral dissociation, they were generally cohesive. Furthermore, despite the prominent nuclear pleomorphism and atypia, most nuclei were centrally positioned within the cells, and the cytoplasmic margins around them were discernible. Although subtle and easily overlooked, these findings, in retrospect, were more consistent with squamous metaplasia than with true malignancy.

Pancreatic juice cytology has been performed in cases where the lesion is not clearly visible on imaging or is complicated by acute pancreatitis, with relatively high diagnostic sensitivity [13,14]. Repeated cell sampling via endoscopic pancreatic duct drainage and stenting allows for examination of more cells, improving the diagnostic accuracy. Sagami et al. [13] examined pancreatic juice cytology in 13 cases and reported that those with a greater number of positive results were more likely to represent true positives, whereas there were fewer positive find-

ings in cases with false-positive results. In our case, pancreatic juice cytology was submitted 15 times over a period of 11 days. Three of the samples were diagnosed as suspicious for malignancy and one as positive for malignancy. Squamous metaplastic cells arise in the pancreatic ducts, so are likely to appear in pancreatic juice cytology. Therefore, the presence of atypical squamous metaplastic cells should be regarded as a cytological finding of particular concern in pancreatic juice specimens.

In conclusion, squamous metaplasia can occur in patients with chronic and/or acute pancreatitis and even in those “without pancreatitis,” occasionally displaying atypia and leading to cytological findings that may mimic adenocarcinoma. Recognition of these cells as squamous metaplastic cells in cytology is extremely challenging because of the lack of keratinization. When cytological features raise suspicion for adenocarcinoma but are not definitive and no mass lesion is detected on imaging, the possibility of benign reactive atypical cells, such as atypical squamous metaplastic cells, should be carefully considered, especially in pancreatic juice cytology.

Ethics Statement

This case report is exempt from ethical review, in accordance with the ethical guidelines of the Japanese Society of Pathology and the institutional policy, as it involves a single case and contains no personally identifiable information. According to the ethical policy of the Japanese Society of Pathology and the policy of our institutional ethics committee, informed consent is not required for case reports if the patient's identity cannot be determined. This report was prepared in compliance with these guidelines, and all identifying information has been removed or anonymized to protect the patient's privacy.

Availability of Data and Material

All data generated or analyzed during the study are included in this published article (and its supplementary information files).

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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Phenotypic plasticity in plasma cell myeloma: a CD138-negative case with a rare *BRAF* G469R mutation

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CD138-negative plasma cell myeloma harboring a *BRAF* G469R mutation is described in a 76-year-old male presenting with multiple osteolytic lesions. Histologically, the lesion exhibited epithelioid to plasmacytoid morphology with prominent mitotic activity and vascular-like spaces. Immunophenotyping demonstrated strong vimentin and CD31 expression but absence of CD138 and other endothelial markers. Light-chain in situ hybridization confirmed a clonal κ -restricted plasma cell population. Bone marrow examination revealed near-complete replacement by atypical plasma cells, retaining CD138 negativity and demonstrating focal CD20 positivity, indicative of intratumoral heterogeneity. Next-generation sequencing identified a rare *BRAF* G469R variant. The patient exhibited poor response to bortezomib, lenalidomide, and dexamethasone therapy, necessitating a switch to carfilzomib-based treatment. This case underscores the diagnostic challenges of CD138-negative myeloma and highlights the importance of integrating morphology, immunophenotyping, and molecular profiling to inform accurate diagnosis and guide therapeutic strategies.

Keywords: Syndecan-1; Multiple myeloma; Plasma cell neoplasms; *BRAF* mutation

INTRODUCTION

Plasma cell myeloma is a neoplasm characterized by the clonal proliferation of terminally differentiated plasma cells, typically expressing syndecan-1 (CD138) as a defining surface marker. CD138 is a heparan sulfate proteoglycan involved in plasma-cell adhesion, differentiation, and secretion, and its expression is considered a hallmark of mature plasma cells [1,2].

However, rare cases of CD138-negative plasma cell myeloma have been reported, presenting significant diagnostic challenges due to their unusual morphology and immunophenotypic profiles that may mimic poorly differentiated sarcomas or hematolymphoid neoplasms of other lineages [3]. The loss or down-regulation of CD138 has been associated with dedifferentiation, increased clonogenic potential, and resistance to chemotherapy, suggesting that this phenotype may represent a biologically distinct and clinically aggressive subset of myeloma [4-9].

In addition, molecular profiling using techniques such as next-generation sequencing (NGS) has been increasingly important in revealing genetic alterations that may contribute to disease heterogeneity and therapeutic response. Among these, *BRAF* mutations, most commonly the V600E substitution, have been identified in a small subset of myelomas, reflecting aberrant mitogen-activated protein kinase (MAPK) pathway activation [10-12]. Nevertheless, non-V600E variants such as *BRAF* G469R remain exceedingly rare, and their clinicopathologic significance in plasma cell neoplasms has not been well characterized.

Herein, we describe a case of CD138-negative plasma cell myeloma harboring a rare *BRAF* G469R mutation, which initially presented with atypical epithelioid morphology and an unusual immunophenotypic profile, posing a significant diagnostic challenge.

This case highlights the importance of combining immuno-

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histochemistry, light-chain in situ hybridization (ISH), and molecular testing in atypical plasma cell neoplasms, and provides further insight into the phenotypic plasticity and molecular diversity of myeloma.

CASE REPORT

A 76-year-old man with a history of hypertension and diabetes presented with progressive left thigh pain for approximately six months. Radiographic examination performed at an outside clinic revealed multiple osteolytic lesions involving the ischium and the subtrochanteric region of the left femur, raising clinical suspicion for a metastatic bone lesion. The patient was referred to our institution for further evaluation and management. Laboratory studies on admission demonstrated anemia (hemoglobin 7.8 g/dL), thrombocytopenia ($103 \times 10^3/\mu\text{L}$), and mild leukopenia (white blood cell count $3.76 \times 10^3/\mu\text{L}$). Serum biochemistry revealed elevated lactate dehydrogenase (264 U/L) and mild hypercalcemia (10.8 mg/dL). Urinalysis showed 30

mg/dL proteinuria, prompting quantitative assessment.

To obtain tissue for diagnosis and to stabilize the bone, curettage of the left femoral lesion was performed. Histologically, the specimen showed large epithelioid to plasmacytoid cells with macronucleoli, stippled chromatin, moderate amphophilic cytoplasm, and frequent mitoses in the background of multiple blood filled cystic spaces (Fig. 1A, B). Immunohistochemically, the tumor cells were negative for CD138, leukocyte common antigen, CD20, CD3, CD30, terminal deoxynucleotidyl transferase, myeloperoxidase, and cytokeratin, but demonstrated strong positivity for vimentin and CD31 (Fig. 1C, D), initially suggesting a vascular tumor. However, additional endothelial markers (CD34, ERG, and D2-40) were negative, and a definitive line of differentiation could not be established at that stage.

During concurrent clinical evaluation, the patient's cytopenias and abnormal protein studies raised clinical suspicion for multiple myeloma (MM). Quantitative urine testing showed 401.5 mg/day of protein, and β_2 -microglobulin was markedly elevated (11.28 mg/L). Urine immunofixation revealed a faint

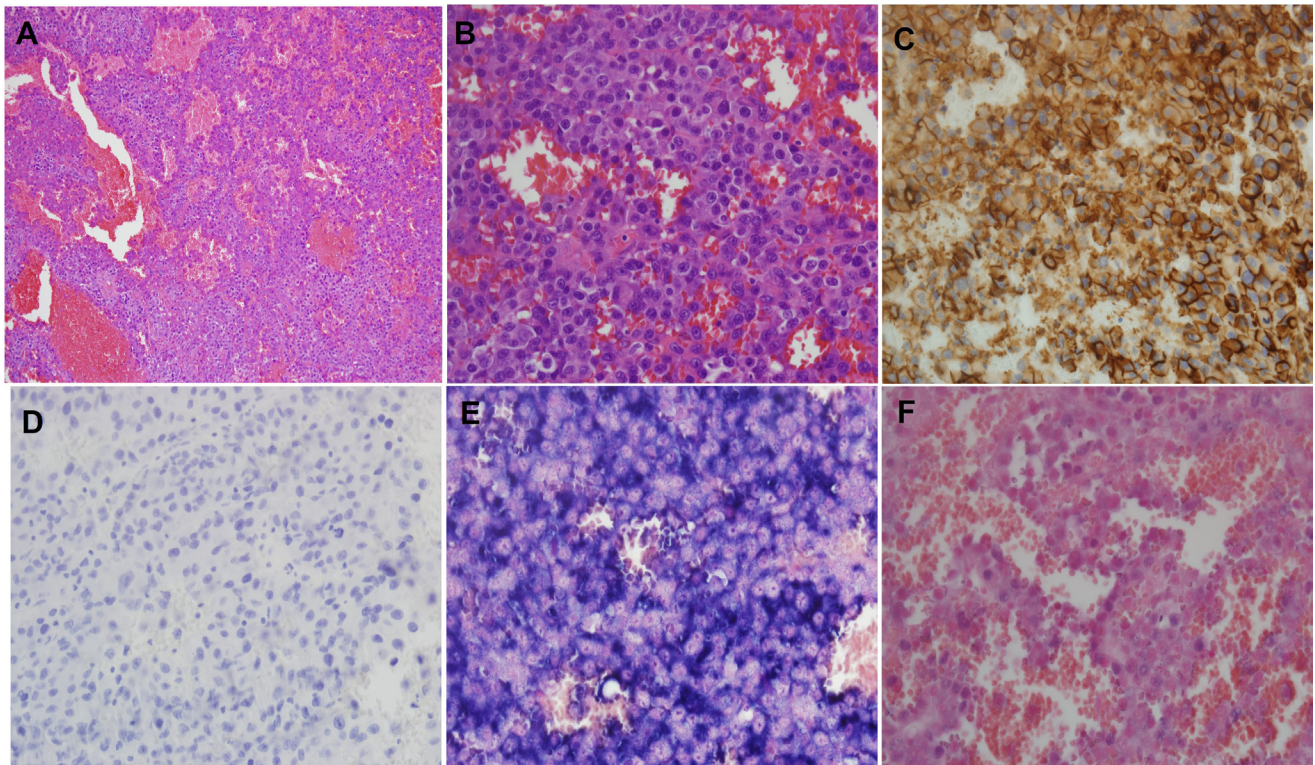


Fig. 1. Histologic and immunohistochemical findings of the femoral lesion. (A) Low-power view showing a solid sheet of tumor cells with multiple red-blood-cell-filled cystic spaces. (B) High-power view demonstrating large epithelioid cells with macronucleoli and amphophilic cytoplasm. (C) Immunostaining for CD31 shows diffuse positivity in tumor cells. (D) CD138 is completely negative. (E) In situ hybridization for κ light chain shows diffuse and strong positivity, confirming κ -restricted clonality. (F) λ light chain is negative.

κ light-chain band, consistent with trace Bence-Jones proteinuria, although the overall urinary protein was predominantly non-monoclonal. In contrast, serum free light chain assay revealed markedly elevated κ light chain at 342.02 mg/L, with λ light chain 14.36 mg/L, resulting in a κ/λ ratio of 23.8, confirming systemic κ -restricted monoclonality.

Given the clinical suspicion of MM, CD138 immunostaining was repeated on multiple tissue sections, but remained negative. ISH for immunoglobulin light chains was subsequently performed, which demonstrated diffuse κ -restricted clonal plasma cell population with complete absence of λ expression (Fig. 1E, F), thus establishing the diagnosis of CD138-negative plasma cell myeloma in the bone lesion.

Subsequent bone marrow biopsy revealed near-total replacement (~99%) of marrow cellularity by atypical plasma cells, confirming advanced-stage MM. Compared with the initial bone lesion, the tumor cells in the bone marrow were morphologically more mature plasma cells (Fig. 2A, B). Immunohistochemically, the tumor cells remained negative for CD138 but demonstrated diffuse κ light-chain positivity on ISH (Fig. 2C, D). Interestingly, CD20 showed focal expression in a subset of

tumor cells, indicating minor phenotypic heterogeneity within the plasma cell population, whereas paired box gene 5 (PAX5) was negative, excluding true B-cell lineage differentiation (Fig. 2E, F).

These findings supported systemic involvement by κ -restricted plasma cell myeloma with partial maturation but persistent loss of CD138 expression. NGS of the bone marrow sample identified a *BRAF* c.1405G>A (p.Gly469Arg) mutation.

The patient was started on bortezomib, lenalidomide, and dexamethasone (VRD) chemotherapy; however, after six cycles, disease progression was observed, and therapy was transitioned to a carfilzomib and dexamethasone regimen.

DISCUSSION

CD138-negative plasma cell myeloma represents a rare and diagnostically challenging subtype of MM. In the present case, the initial biopsy exhibited an epithelioid morphology with vascular-like spaces and an unusual immunophenotypic profile, including vimentin and CD31 expression in the absence of conventional plasma cell markers, which complicated the diag-

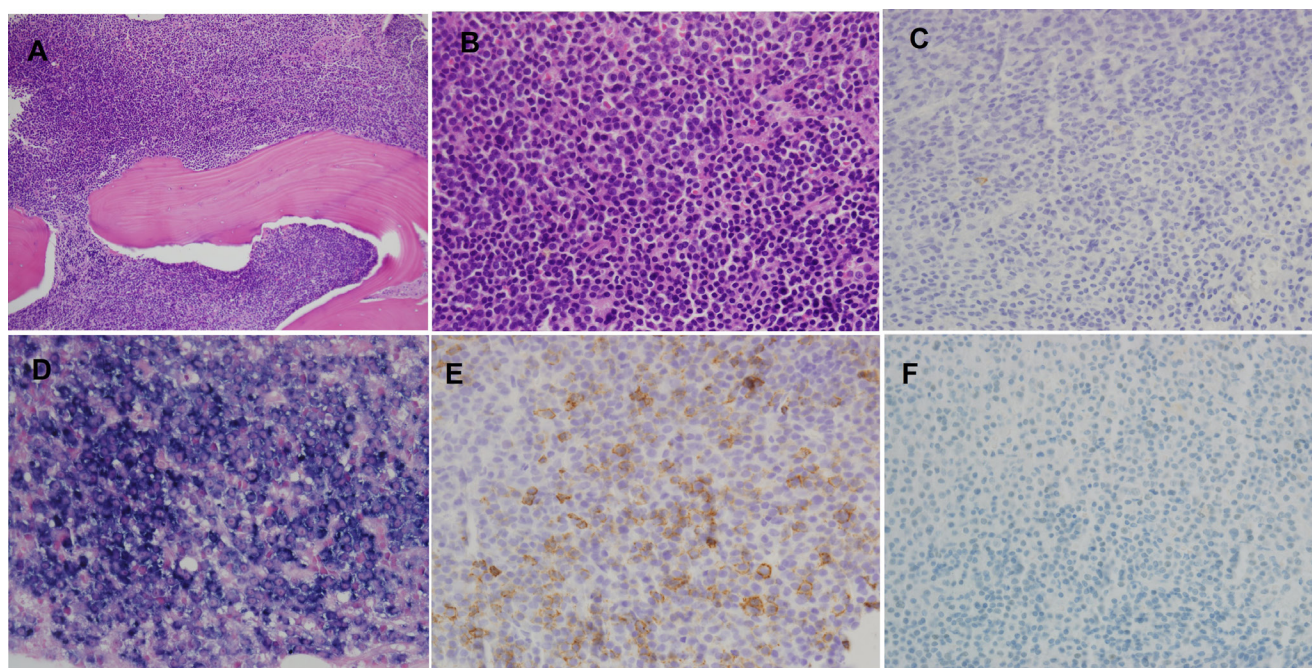


Fig. 2. Bone marrow findings. (A) Diffuse infiltration of the bone marrow by atypical plasma cells, replacing nearly all hematopoietic elements. (B) The tumor cells show relatively smaller size and more mature morphology compared with the femoral lesion. (C) Immunohistochemistry for CD138 shows complete loss of expression. (D) In situ hybridization for κ light chain demonstrates diffuse and strong positivity, confirming κ -restricted clonality. (E) CD20 shows focal cytoplasmic and membranous positivity in a subset of tumor cells, indicating minor phenotypic heterogeneity. (F) PAX5 is completely negative, confirming absence of B-cell lineage differentiation.

nostic process. Ultimately, demonstration of κ -restricted plasma cells by ISH established the diagnosis of CD138-negative plasma cell myeloma, highlighting the importance of ancillary molecular studies in diagnostically ambiguous cases.

CD31 (PECAM-1) is widely recognized as an endothelial marker; however, its expression is not restricted to endothelial cells. Multiple studies show that CD31 is also expressed in reactive and neoplastic plasma cells, plasmablastic variants of myeloma, and other hematopoietic cells such as leukocytes, monocytes, and platelets, highlighting that CD31 positivity alone cannot be used to confirm endothelial lineage in tumors [13]. In plasma cell lesions specifically, CD31 expression has been documented in a high proportion of conventional myelomas and plasmablastic variants, indicating that CD31 expression in plasma cells is a recognized immunophenotypic feature and may confound differential diagnosis with vascular neoplasms if interpreted without additional lineage-specific markers [14].

CD138 (syndecan-1) is a transmembrane heparan-sulfate proteoglycan essential for plasma cell adhesion, differentiation, and secretion [1,2]. Loss or downregulation of CD138 occurs in a subset of myelomas and is associated with an immature, stem-like phenotype and drug resistance [4,7,8]. Experimental studies have shown that CD138-low or -negative myeloma cells exhibit increased clonogenicity, self-renewal potential, and reduced expression of terminal differentiation markers [4-6]. In our patient, the absence of CD138 expression correlated with atypical morphology and poor treatment response. After six cycles of VRD chemotherapy, the disease progressed and required transition to a carfilzomib-based regimen, consistent with the chemoresistant behavior described in CD138-low myelomas [7]. The microenvironmental interactions of CD138-negative cells may further contribute to therapeutic resistance through alterations in stromal stiffness and SDF-1/CXCR4 signaling [9].

Molecular analysis of the bone marrow specimen revealed a *BRAF* G469R mutation, a rare non-V600E variant within the kinase domain. *BRAF* mutations are functionally classified into three categories based on their mechanism of MAPK pathway activation [10,11]. Class I mutations, typified by V600E, signal as RAS-independent monomers with high kinase activity. In contrast, class II mutations, including G469 substitutions, activate downstream signaling through RAS-independent dimerization, while class III mutations exhibit impaired kinase activity and rely on upstream RAS activation. Accordingly, *BRAF* G469R is classified as a Class II mutation, leading to constitutive MAPK pathway activation via dimer-dependent

signaling.

In MM, *BRAF* mutations are reported in a small subset of cases, with V600E being the most common variant. Non-V600E mutations such as G469R are exceedingly rare, and their clinicopathologic significance remains poorly defined [12]. Limited available data suggest that MAPK pathway activation through non-V600E *BRAF* mutations may contribute to disease progression, clonal evolution, and therapeutic resistance. In other malignancies, including non-small cell lung carcinoma, melanoma, and colorectal carcinoma, *BRAF* G469 mutations have been associated with limited responsiveness to *BRAF* inhibitor monotherapy, whereas downstream inhibition with MEK or ERK inhibitors may offer greater therapeutic potential [10,11]. Although targeted therapy was not applied in the present case, the identification of a class II *BRAF* mutation raises the possibility that aberrant MAPK signaling contributed to the aggressive clinical course and resistance to conventional therapy.

Interestingly, focal CD20 positivity was observed in the bone marrow lesion, whereas the initial femoral lesion was CD20 negative. This finding suggests phenotypic heterogeneity within the tumor and may reflect clonal evolution during disease progression. CD20-positive myelomas are uncommon, detected in roughly 10%–20% of cases, and have been associated with t(11;14)(q13;q32) translocation, lymphoplasmacytoid morphology, and sometimes adverse prognosis [15]. However, the clinical implications remain controversial. In our case, despite focal CD20 expression, PAX5 was negative, indicating that the CD20-positive population does not represent true B-cell lineage differentiation. Rather, this pattern may reflect partial re-expression of B-cell antigens during clonal evolution or phenotypic drift within the plasma cell clone. Such partial immunophenotypic reversion, combined with the absence of CD138, supports the concept of plasticity and dedifferentiation in certain myeloma subclones [6,7,9,15]. This dynamic immunophenotypic change might also underlie the chemoresistant and stem-like behavior described in CD138-low or -negative myelomas.

In conclusion, CD138-negative plasma cell myeloma represents a biologically distinct phenotype characterized by diagnostic ambiguity, reduced secretory activity, and relative resistance to therapy. Recognition of this variant and application of light-chain ISH and molecular testing are crucial for accurate diagnosis. Future studies correlating immunophenotypic variability (including CD20 and CD138) with genomic drivers such as *BRAF* mutations may clarify mechanisms of dedifferentiation and identify potential therapeutic targets.

Ethics Statement

This study was approved by the Institutional Review Board of Kosin University Gospel Hospital with a waiver of informed consent (IRB No. 2025-10-029).

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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SDH-deficient renal cell carcinoma with intracytoplasmic mucinous material: a case report and literature review

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Succinate dehydrogenase (SDH)-deficient renal cell carcinoma (RCC) is a rare, molecularly defined neoplasm. We report a 45-year-old man with a right renal mass treated by nephrectomy. Grossly, a 38-mm gray-white-to-brown solid cystic tumor was observed in the lower pole of the kidney. Microscopically, the tumor consisted of sheets and nested proliferation of eosinophilic cells with low-grade nuclei and bubbly or flocculent cytoplasm. No sarcomatoid or rhabdoid features were observed. Abundant extracellular and focal intracytoplasmic mucinous material was observed in the tumor, which was positive for Alcian blue and mucicarmine staining, but negative for periodic acid-Schiff staining. Immunohistochemistry showed complete loss of succinate dehydrogenase subunit B in tumor cells. These findings supported the diagnosis of RCC consistent with SDH-deficient RCC. This case expands the morphological spectrum of SDH-deficient RCC and highlights the diagnostic pitfalls of renal tumors with mucinous material.

Keywords: Carcinoma, renal cell; Kidney neoplasms; Succinate dehydrogenase; Immunohistochemistry; Genetic testing

INTRODUCTION

Succinate dehydrogenase (SDH)-deficient renal cell carcinoma (RCC) is a molecularly defined RCC, comprising less than 0.2% of all RCCs and typically affecting younger individuals, with a median age of approximately 35 years at diagnosis. The SDH complex, composed of four subunits (SDHA, SDHB, SDHC, and SDHD), is a mitochondrial enzyme integral to both the Krebs cycle and electron transport chain. Germline mutations in any of these subunits can lead to destabilization of the SDH complex, resulting in the loss of SDHB protein expression—a diagnostic hallmark of SDH-deficient tumors [1].

SDH-deficient RCC is histologically characterized by nests or sheets of uniform eosinophilic cells with indistinct borders, low-grade nuclei, and cytoplasmic vacuoles or inclusions con-

taining pale eosinophilic or flocculent material [1]. Although most tumors exhibit indolent morphology, recent studies have highlighted a broader histological spectrum, including high-grade transformation, sarcomatoid or rhabdoid features, and overlapping morphology with fumarate hydratase (FH)-deficient RCC and transcription factor EB (TFEB)-rearranged RCC [2-5].

To date, mucinous material has not been recognized as a common morphological feature of SDH-deficient RCC, and reports describing this phenomenon remain limited [2,6]. Herein, we report a case of RCC consistent with SDH-deficient RCC showing focal intracytoplasmic mucinous material. This case expands the recognized morphological heterogeneity of this rare tumor subtype. Awareness of this atypical presentation is essential to avoid misclassification—particularly in the

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differential diagnosis of mucin-producing renal neoplasms and metastatic carcinomas—and to prompt appropriate ancillary testing, including SDHB immunohistochemistry and genetic evaluation.

CASE REPORT

A 45-year-old Japanese man with no notable medical history, including a history of malignancy or hereditary syndromes, was incidentally diagnosed with a right renal mass during a routine health screening. The patient's mother had died of colon cancer, and the histopathological subtype was unknown. Abdominal imaging revealed a 40-mm mass in the lower pole of the right kidney. Contrast-enhanced X-ray computed tomography showed early enhancement with washout, and magnetic resonance imaging revealed a partially cystic lesion with high signal intensity on T2- and diffusion-weighted imaging. Preoperatively, no clear evidence of extrarenal invasion or metastasis was observed. A laparoscopic right nephrectomy was performed for the clinical diagnosis of RCC.

The resected specimen showed a well-circumscribed, gray-white-to-dark-brown, solid, hemorrhagic, and cystic tumor measuring 38 × 30 × 25 mm in the renal parenchyma (Fig. 1A). Microscopically, the tumor displayed predominantly sheet-like and nested growth patterns with focal cystic areas. At the tumor periphery, no distinct fibrous capsule was observed, and entrapped non-neoplastic renal tubules were noted (Fig. 1B). The tumor cells had bland, round-to-oval nuclei, corresponding to World Health Organization/International Society of Urological Pathology nuclear grade 1, and an eosinophilic cytoplasm with frequent bubbly or pale flocculent features (Fig. 1C). The cell borders were indistinct. Although cystic degeneration and hemorrhage were present, no evidence of coagulative tumor necrosis was observed. No high-grade features, such as sarcomatoid or rhabdoid changes, nuclear pleomorphism, or increased mitotic activity, were identified. Notably, the mucinous material was distributed in a patchy pattern throughout the tumor, predominantly in the intercellular stroma; however, focal and distinct intracytoplasmic accumulation was also observed (Fig. 1D). The intracytoplasmic mucinous material was positive for Alcian blue (pH 2.5) (Fig. 1E) and mucicarmine staining, but negative for periodic acid–Schiff (PAS) staining (Fig. 1F). The mucinous material distributed in the intercellular stroma was also positive for Alcian blue, but negative for mucicarmine staining.

Immunohistochemically, the tumor cells were positive for PAX8 and focally positive for cytokeratin (CK) AE1/AE3, alpha-methylacyl-CoA racemase, CD117, and vimentin, and negative for CK7, CD10, RCC markers, human melanoma black 45, and anaplastic lymphoma kinase (ALK). Transcription factor E3 (TFE3) showed cytoplasmic staining only without nuclear expression, and TFEB showed only weak staining without evidence of overexpression. SDHB immunostaining showed a complete loss of cytoplasmic expression in the tumor cells, with strong granular positivity in the adjacent non-neoplastic renal tubules, serving as an internal positive control (Fig. 2A). SDHA expression was retained in the tumor cells (Fig. 2B). Genetic testing for pathogenic germline variants in the SDH genes (*SDHA*, *SDHB*, *SDHC*, and *SDHD*) was not performed because the patient did not provide consent.

Based on the morphologic and immunohistochemical findings, the tumor was considered consistent with SDH-deficient RCC with focal intracytoplasmic mucinous material. The tumor was confined to the kidney, with no invasion into the renal sinus or perinephric fat. Lymphovascular invasion of tumor cells was not identified, and the surgical margins were negative. The pathologic T category was pT1a. The patient remains alive and well, with no evidence of recurrence or metastasis at 49 months postoperatively.

DISCUSSION

RCC encompasses diverse subtypes, defined by distinct morphologies, immunophenotypes, and molecular alterations. SDH-deficient RCC is a rare subtype that typically arises in younger individuals and is characterized by loss of SDHB protein expression [1]. In the present case, the unilateral renal mass displayed morphologic features compatible with SDH-deficient RCC, including an eosinophilic cytoplasm with a bubbly or flocculent appearance, low-grade nuclei, and a heterogeneous architecture composed of sheet-like and microcystic patterns. Notably, Alcian blue-positive mucinous material was found extracellularly and focally within the cytoplasm, which is an uncommon feature of SDH-deficient RCC.

Recently, the morphological spectrum of SDH-deficient RCC has expanded. In addition to the classical bland histology, some tumors exhibit overlapping features with FH-deficient RCC or TFEB-rearranged RCC or display high-grade transformations such as sarcomatoid or rhabdoid differentiation. These variant morphologies may correlate with more aggressive clinical be-

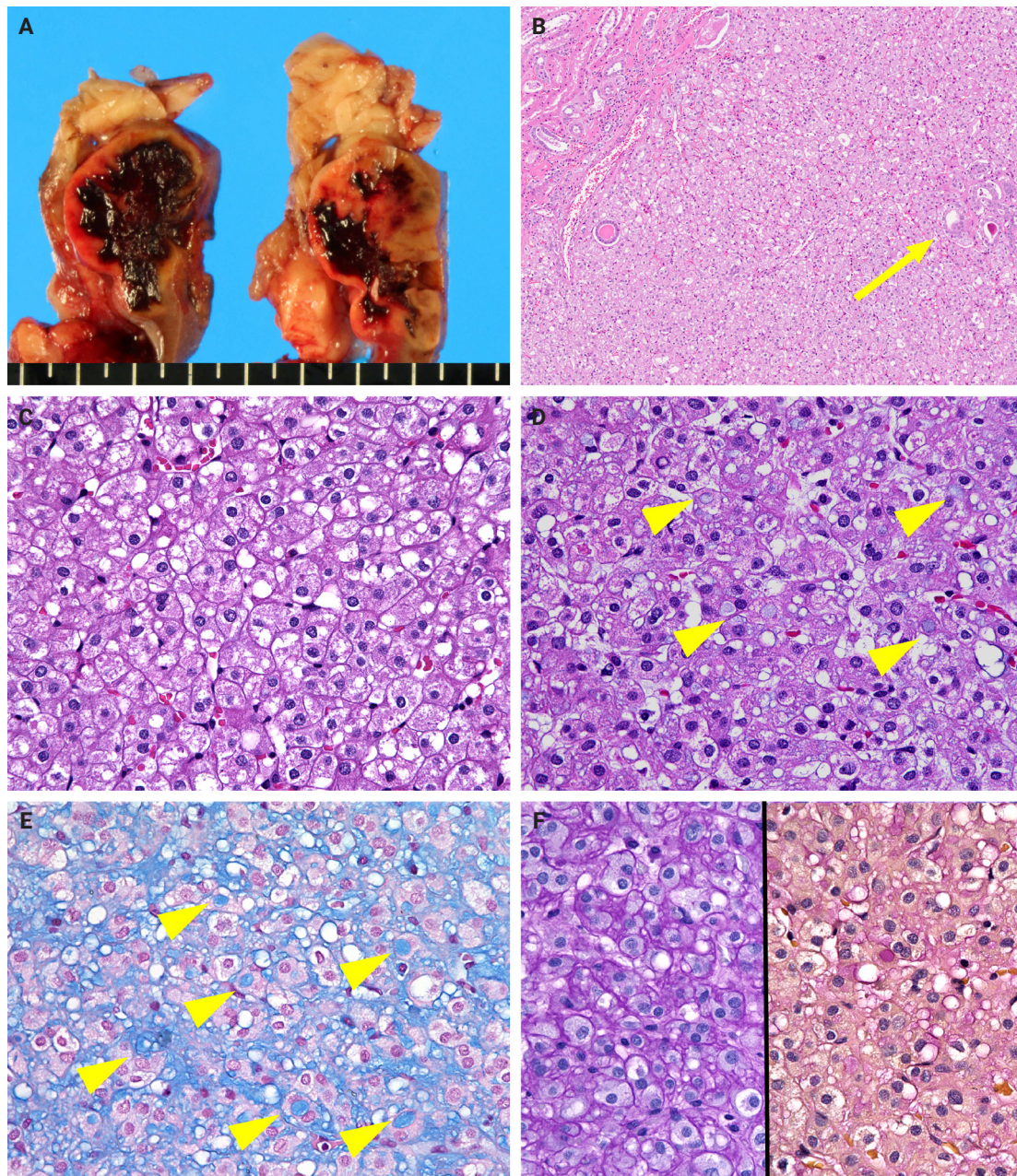


Fig. 1. Gross and histologic findings. (A) The cut surface of the resected right kidney shows a well-circumscribed, gray-white-to-dark-brown tumor with solid, hemorrhagic, and cystic areas. (B) No distinct fibrous capsule is observed at the tumor periphery. Entrapped non-neoplastic renal tubules are detected in the tumor (arrow). (C) Tumor cells consist of low-grade nuclei and bubbly or flocculent, eosinophilic cytoplasm. (D) Intracytoplasmic mucinous material is occasionally detected in tumor cells (arrowheads). (E, F) The intracytoplasmic mucinous material is positive for Alcian blue (pH 2.5) staining (arrowheads) (E), and mucicarmine staining (F, right), and negative for periodic acid-Schiff (PAS) staining (F, left).

havior [2-5]. Only two cases of mucinous material deposition in SDH-deficient RCC have been reported to date. Ajmal et al. described the presence of extracellular mucinous material in a high-grade tumor with sarcomatoid and rhabdoid features.

That study noted only a small amount of extracellular mucinous material, and no histological images were provided. Additionally, no information was provided about the distribution pattern of mucinous material deposition or the results of the

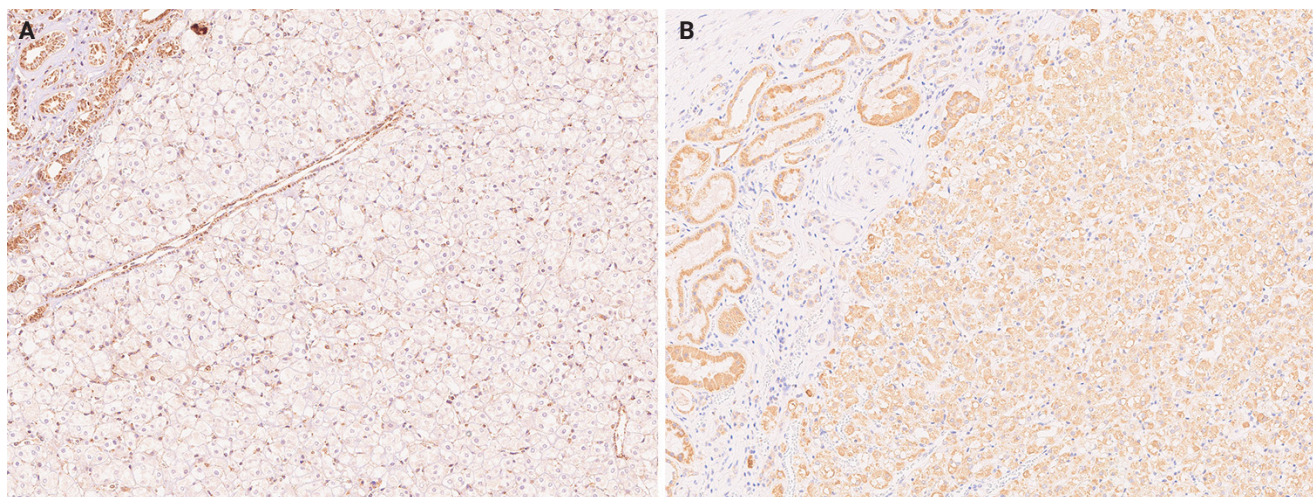


Fig. 2. Immunohistochemical findings. (A) Tumor cells show loss of succinate dehydrogenase subunit B (SDHB) expression. Note the strong cytoplasmic immunoreactivity in adjacent non-neoplastic renal tubules and interstitial cells, serving as internal positive controls. (B) Succinate dehydrogenase subunit A (SDHA) immunohistochemistry demonstrates retained diffuse granular cytoplasmic staining in tumor cells and non-neoplastic elements.

histochemical staining used to determine its nature [2]. Hu et al. [6] reported intracellular and intraluminal mucinous material deposition in SDHA-deficient RCC. That study described single tumor cells containing intracellular mucinous material and small- to medium-sized, round, dilated, and twisted glandular ducts around the tumor nodule. Histologic images showed gray-blue mucinous material filling the lumina of the tumor glands. However, no information was provided regarding the results of histochemical staining [6]. In contrast, our case demonstrated Alcian blue-positive, PAS-negative mucinous material predominantly located in the intercellular spaces, with focal intracytoplasmic deposition, indicating the presence of acidic mucopolysaccharides. Furthermore, mucicarmine staining was weakly and focally positive, which supports this interpretation. To the best of our knowledge, this case represents one of the few reported cases in which the mucinous material was characterized histochemically. This finding expands the known morphological spectrum of this tumor type and underscores the importance of recognizing the deposition of mucinous material in SDH-deficient RCC. Further accumulation of cases with detailed histochemical evaluation is required to clarify the nature and distribution of mucinous material.

The differential diagnosis in the present case includes other renal neoplasms with eosinophilic cytoplasm and mucinous material. Among renal tumors, mucinous tubular and spindle cell carcinoma (MTSC) is an important consideration because

it characteristically shows mucinous stroma. However, the present tumor lacked the spindle cell component and well-formed tubular structures that are characteristic of MTSC, and was also negative for CK7 and CD10, which is inconsistent with MTSC. ALK-rearranged RCC may also show extracellular and intracellular mucinous material, sometimes with signet-ring-like change [7]. However, the present tumor was negative for ALK and lacked other characteristic features, such as inflammatory cell infiltration and papillary or cribriform architecture. TFE3/TFEB-rearranged RCC was also considered, given its known morphologic variability and occasional overlapping eosinophilic features. However, the present tumor showed no obvious histologic findings including biphasic clear/eosinophilic cell population and basement membrane-like material, or convincing TFE3/TFEB overexpression; therefore, a TFE3/TFEB-associated translocation renal cell carcinoma was not supported. In addition, PAX8 positivity supported renal epithelial origin, arguing against non-renal SDH-deficient neoplasms such as paraganglioma. Taken together, the morphologic and immunohistochemical findings supported a diagnosis of RCC consistent with SDH-deficient RCC.

Loss of SDHB expression is a diagnostic hallmark of SDH-deficient tumors, irrespective of the subunit affected. Dysfunction of any SDH subunit (*SDHA*, *SDHB*, *SDHC*, or *SDHD*) disrupts the complex, causing the SDHB protein to become unstable and undetectable. Therefore, SDHB immunohistochemistry

serves as a reliable diagnostic marker of SDH deficiency [8]. In the present case, the combination of complete loss of SDHB expression and retained SDHA expression was diagnostically important and strongly supported SDH-deficient RCC. At the same time, this immunophenotype does not identify the specific underlying SDH gene alteration. Among SDH-deficient RCCs, SDHB-deficient RCC is predominant, whereas SDHA-, SDHC-, and SDHD-deficient RCCs are less common. Despite limited data, tumors with SDHA alterations tend to show unusual morphologies, including high-grade nuclear atypia, papillary, cribriform, sarcomatoid, and rhabdoid morphologies [1,6]. Interestingly, the only previously documented case of intracytoplasmic mucinous material accumulation in SDH-deficient RCC was also associated with SDHA deficiency [6]. In contrast, the retained SDHA expression in the present case argues against SDHA-deficient RCC with loss of SDHA protein expression. However, because molecular genetic testing was not performed, the precise SDH subunit alteration could not be determined. Further accumulation of genetically characterized cases will be necessary to clarify whether specific SDH subunit alterations are associated with distinct morphologic features or clinical behavior.

Germline alterations in SDH subunit genes underlie SDH-deficient tumor syndrome, which encompasses pheochromocytoma/paraganglioma, SDH-deficient gastrointestinal stromal tumors, and pituitary neuroendocrine neoplasms. Although immunohistochemical SDHB loss in tumor cells is an effective marker of SDH deficiency, it cannot differentiate between mutated SDH subunits (except that concurrent loss of SDHB and SDHA suggests SDHA deficiency) [8]. A major limitation of the present case is the absence of molecular genetic testing. Although the morphologic findings and immunophenotype, particularly SDHB loss with retained SDHA expression, strongly support SDH-deficient RCC, the specific underlying genetic alteration could not be identified. However, genetic testing is generally recommended because identifying the specific SDH subunit allows for the customization of surveillance [9]. Each subunit is associated with a different set of tumors and different penetrance, and the result enables targeted cascade testing in relatives. Importantly, genotyping also distinguishes *SDHB*-, *SDHC*-, and *SDHD*-mutated tumors that are indistinguishable by immunohistochemistry, thereby facilitating clinicopathological correlations, including comparisons of morphological variation and clinical course across subtypes. Even when genetic testing is not performed, long-term surveillance for other

SDH-deficient neoplasms should be considered [9].

In summary, we report one of the few documented cases of RCC consistent with SDH-deficient RCC showing histochemically characterized extracellular and focal intracytoplasmic mucinous material. This case further expands the morphological spectrum of SDH-deficient RCC. SDH-deficient RCC should be considered in the differential diagnosis of renal tumors with mucinous material, although such cases are likely rare. Further case accumulation with genetic analysis of the SDH subunit genes is warranted to clarify the correlation between specific SDH subunit mutations and distinct histological features.

Ethics Statement

This single case report was exempted from Institutional Review Board review and subsequent informed consent by the National Defense Medical College, Tokorozawa, Japan (Registration number, 5156; decision date, March 10, 2025).

Availability of Data and Material

Data sharing not applicable to this article as no datasets were generated or analyzed during the study.

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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