POU2F3 beyond thymic carcinomas: Expression across the spectrum of thymomas hints to medullary differentiation in type A thymoma

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Abstract (218 words)

The thymic medulla comprises various cell types, including tuft cells that are involved in innate immunity. We recently reported that in Western cohorts of patients, most thymic squamous cell carcinomas (TSQCCs), in contrast to thymomas, exhibit strong and extensive expression of tuft cell markers, including the tuft cell master regulator, POU2F3. On closer inspection of 94 thymomas that cover the full spectrum of thymoma histotypes, we now find by immunohistochemistry that approximately half of type A, AB, and B1 thymomas contain small numbers (<10%) of cells expressing POU2F3, while most type B2 and B3 thymomas do not ($p < 0.05$). Further, in rarer type A and AB thymomas with adenoid growth pattern, POU2F3(+) cells formed aggregates and co-expressed KIT, as did the tumor cells in 100% (9/9) of TSQCCs expressing POU2F3. However, the expression of another tuft cell marker, L1CAM, still distinguished TSQCC from the spectrum of thymomas that were all L1CAM-negative. This study is the first to demonstrate the high frequency of POU2F3 expression in an Asian cohort of TSQCCs. The common occurrence of scattered POU2F3(+) cells in type A and AB thymomas, hints at their variable degree of medullary differentiation and supports the historical hypothesis of the medullary nature of type A thymomas. Immunohistochemistry of L1CAM may be a valuable tool to differentiate TSQCCs from thymomas.

Keywords:

Thymus; Thymic epithelial tumors; Thymic tuft cells; POU2F3; L1CAM

Text (2537 words)

Introduction

A deeper knowledge of the cellular components of normal tissues and organs will advance the understanding and potential treatment of cancers that develop in the corresponding regions. Single-cell RNA sequencing studies have revealed unexpected cellular heterogeneity in many organs [10, 16]. The thymus and thymic epithelial cells (TECs) are no exception, and have been found to consist of diverse cellular components, including thymic tuft cells [4, 15].

Tuft cells are epithelial cells characterized by unique long and thick microvilli (tufts) on their apical side. These cells are linked to type 2 immunity in the small intestine [7, 9, 20, 25], where they serve as sensors for various chemical signals (including those from parasites), and through the secretion of biological mediators, initiate anti-parasitic immune responses. Thymic tuft cells, which were discovered as members of a diverse population of medullary TECs (mTECs), shape the microenvironment and influence innate immunity [4, 15].

We hypothesized that this heterogeneity of non-neoplastic TECs might explain the morphological and molecular diversity of thymic epithelial tumors (TETs) [19] and found that most thymic carcinoma, especially thymic squamous cell carcinoma (TSQCC), exhibited significantly increased expression of tuft cell-related genes, including POU2F3, the master regulator of tuft cells [29], on mRNA and protein levels [27]. This was the second report about tuft cell-like carcinoma following the discovery of a tuft cell-like variant in small cell

lung cancer (SCLC) [11].

Although POU2F3 expression was significantly higher in thymic SQCC than other TET subtypes, we noticed that some thymomas contained POU2F3-positive cells, albeit far fewer (less than 10% among tumor cells and clearly under the cutoff value of the previous study [40%] [27]) than tuft cell-like thymic SQCC. In the present study, we investigated the distribution of these rare POU2F3-positive cells in thymomas in detail in order to achieve a better understanding of TETs, especially their histogenesis. We also performed L1CAM immunohistochemistry (IHC) because *L1CAM* is also a thymic tuft cell-related gene with significantly stronger expression in thymic carcinomas than thymomas at the mRNA level [5, 6, 27], suggesting that its expression status might lead to more accurate diagnosis in TETs or confer new insights into the link between the tuft cell phenotype and thymic tumors.

Materials and Methods

Case selection

Among TET cases archived in Kyoto University Hospital (approximately 260 cases between 1998 and 2021), we examined a total of 103 cases for which tissue microarrays (TMAs) with a 2 mm core size had been established. All of the cases were surgically resected between 2002 and 2015. Because cases of micronodular thymoma with lymphoid stroma (MNTs) were not included in these TMAs, we examined all MNTs surgically resected between 2016

and 2021 (n = 8) with whole slide sections. Thus, a total of 111 TETs were analyzed.

Immunohistochemistry

IHC was performed on formalin-fixed, paraffin-embedded specimens using an automated immunostainer (Benchmark Ultra, Ventana Medical Systems, Oro Valley, AZ, USA). The primary antibodies used were those against CD5 (clone 4C7; Leica Biosystems, Wetzlar, Germany), Ki-67 (clone MIB-1; Agilent Technologies), KIT (polyclonal, Agilent Technologies), L1CAM (clone EPR18750; Abcam, Cambridge, UK), and POU2F3 (polyclonal, Sigma-Aldrich, St. Louis, MO, USA). Renal tubules were used as a positive control for L1CAM (Figure S1) (The Human Protein Atlas [proteinatlas.org]).

The IHC for POU2F3 and L1CAM was interpreted as positive when 10% or more of the tumor cells exhibited nuclear (POU2F3) and membranous (L1CAM) stainings in a hotspot, i.e., in one optical field at 200x magnification. When less than 10% of tumor cells exhibited positivity, it was interpreted as "scattered" because POU2F3-positive cells were dispersed without aggregations in such cases. For thymoma cases, the number of POU2F3-positive cells (henceforth labeled POU2F3[+]) in a hot spot was also evaluated.

Ten representative cases that contained POU2F3(+) cells (type A thymoma, 3; AB, 3; B1, 3; B3, 1) were further pathologically analyzed with whole sections to estimate the spatial distribution of POU2F3(+) cells in the type A and B-like components as well as medullary

islands (MIs) of type AB thymoma, and the MIs and cortical regions of type B1 thymomas.

Statistical analysis

The difference in categorical variables was evaluated by the chi-square test, and that of continuous variables was evaluated by the Wilcoxon test. p values < 0.05 were considered significant.

Results

Clinical findings

The number of male and female patients was 52 and 59, respectively. Patient age ranged from 19 to 86 years old, with a median of 59.5 years old.

Histological classification

Before performing IHC analyses, all cases were reviewed and subclassified according to the current World Health Organization classification [3] as follows: type A thymomas, 12; AB, 28; B1, 7; B2, 24; B3, 14; thymic carcinoma, 17 (SQCC, 16; mucoepidermoid carcinoma, 1); micronodular thymoma with lymphoid stroma, 8; metaplastic thymoma, 1.

Abundance and immunophenotype of POU2F3-positive cells in TETs

Most thymic carcinomas (9/17 [53%]; 9/16 [56%] of SQCC and 0/1 [0%] of mucoepidermoid carcinoma) were positive for POU2F3, i.e., harbored over 10% POU2F3(+) cells, while all thymomas except for one type A thymoma (see below) (1/87, 1%) were labeled negative. However, approximately half of the other type A (5/12, 42%), AB (20/29, 69%), and B1 (3/7, 43%) thymomas contained scattered POU2F3(+) cells below the 10% threshold (Figure 1a-f and S2a-f). Because only one of 14 type B3 thymomas (7%) and no type B2 thymomas (0/23, 0%) contained POU2F3(+) cells, the frequency of POU2F3(+) cells differed significantly between type A, AB, and B1 thymoma on the one hand and type B2 and B3 thymomas on the other ($p < 0.05$) (Figure 2a). All eight micronodular thymomas, from which whole slide sections were examined, contained scattered POU2F3(+) cells (8/8 [100%]) (Figure S2e-f). One metaplastic thymoma did not contain POU2F3-positive cells (0/1 [0%]) (not shown). For the thymoma cases with POU2F3(+) cells, these numbers were not significantly different among the five histotypes ($p = 0.23$) (type A, 1-255 [median 4.5]; type AB, 1-72 [median 10.5]; type B1, 2-40 [median 3]; type B3, 2; MNT, 4-78 [median 35]). In each thymoma type, tumor stage (TNM and Masaoka) and the complication rates of myasthenia gravis were not statistically different between cases with or without POU2F3(+) cells (not shown).

One type A thymoma exhibited adenoid growth pattern, and only this case contained small clusters of POU2F3-positive cells that, surprisingly, co-expressed KIT as do tuft cell-like thymic SQCC [27] (Figure 3a-d). Unlike thymic SQCC, however, CD5, another

classic thymic carcinoma marker [8] was negative in these cells, and the Ki-67 labeling index was almost 0% (Figure 3e-f). Similarly, in a rare type AB thymoma with an adenoid type A component archived in another institute (University Medical Centre Mannheim, Germany), the adenoid component also contained POU2F3 (+)/KIT (+)/CD5(-) tumor cell clusters (unpublished data) (Figure S3a-b).

Spatial distribution of POU2F3-positive cells in TETs

Because TETs, especially type AB thymomas, can exhibit complex histological features within individual tumors [3], we analyzed the spatial distribution of POU2F3(+) cells in type A (n = 3), AB (n = 3), B1 (n = 3), and B3 (n = 1) thymomas with whole slide sections.

Although it was not statistically assessed, the following tendency was observed. The distribution of POU2F3(+) cells in thymoma was generally heterogeneous irrespective of histotypes, including those which typically show neither MIs nor other distinct compartments [3] (Figure S4a). In type AB thymomas, POU2F3(+) cells occurred in all of type A, type B-like components, and MIs (if present). The number or density in each area was variable in each case, but the density in type A components and in MIs often seemed higher than in type B-like components (Figure S4b). In type B1 thymomas, POU2F3(+) cells were generally easily observed in MIs (in the 3 cases), but in 1 case also occurred additionally outside (Figure 1a-b).

156

157 ***L1CAM-positive cells in thymic epithelial tumors***

158 IHC was performed for L1CAM, another thymic tuft cell marker [4, 17], based on the
159 expectation that thymic carcinomas would significantly express this marker, in parallel with
160 POU2F3. Consistent with this hypothesis, 6 of 17 (35%) thymic carcinomas (all were
161 TSQCCs) were positive for L1CAM, while all types of thymomas were negative (0/94, 0%; p
162 < 0.05) (Figure 2b and 4a-f).

163 Unexpectedly, expression of POU2F3 and L1CAM was poorly correlated; among 17
164 thymic carcinomas, only 2 cases (12%) were positive for both markers, 7 cases were positive
165 only for POU2F3, 4 cases were positive only for L1CAM, and 4 cases were negative for both
166 markers. Since all thymomas were L1CAM-negative, their POU2F3(+) cells did not
167 co-express L1CAM (not shown). This result could be of diagnostic value because 13 of 17
168 (76%) thymic carcinomas were positive for POU2F3 and/or L1CAM while all of the
169 thymomas, except for the type A thymoma with POU2F3 (+)/KIT (+)-clusters, were negative
170 for both markers (85/86, 99%) (Figure 2c).

171

172 **Discussion**

173 This study provides new insights into POU2F3(+) cells in thymic epithelial tumors,
174 particularly it shows that approximately half of type A, AB, and B1 thymomas and virtually

all MNTs contain POU2F3(+) cells, albeit in much lower numbers than thymic carcinomas. Aside from a study that reported the high prevalence of a tuft cell-like signature in thymic carcinoma [27], we recently found that multilocular thymic cysts (MTCs), non-neoplastic lesions that supposedly arise from mTECs, can contain POU2F3(+) cells, probably thymic tuft cells within the epithelium [22]. Accordingly, our current understanding of POU2F3(+) cells in thymic epithelial lesions is summarized in Figure 5.

Considering the morphology of MIs, which sometimes contain Hassall's corpuscles, it is reasonable to think that POU2F3-positive cells in MIs in type B1 and AB thymomas have properties of thymic tuft cells. The significance of POU2F3-positive cells outside of MIs in type B1 thymoma is inconclusive but might imply aberrant medullary differentiation at the single-cell level. Considering the preferred location of POU2F3-positive cells in the thymic medulla and medulla-like regions in normal, reactive, and some neoplastic conditions (i.e., MIs in thymomas), we speculate that POU2F3 can be regarded as an mTEC marker in TETs.

Pathologists have attempted to elucidate the histogenesis or cell-of-origin of thymomas for a long time. The cortical phenotype of type B thymomas (especially B2 thymomas) is generally accepted [18, 21, 28], and there is strong evidence for bi-lineage, that is, corticomedullary differentiation, for type AB and B1 thymomas [21]. In contrast, the histogenesis of type A thymoma has remained enigmatic because lacking expression of previously examined *bona fide* cortical and medullary markers (e.g., β5T, PRSS16, AIRE,

194 CD40, involucrin) did not allow conclusions to be drawn [21]. To our knowledge, the current
195 study is the first to demonstrate the expression of a gene restricted to the normal thymic
196 medulla in type A thymomas, supporting the historical hypothesis of the medullary nature of
197 type A thymomas [13].

198 All eight micronodular thymomas exhibited scattered, heterogeneously distributed
199 POU2F3(+) cells; The high frequency of MNTs expressing rare POU2F3(+) cells might be
200 due to the analysis of whole slides instead of TMA cores. This finding confirms the close
201 relationship between type A thymomas and MNTs, which was previously suspected based on
202 morphological and molecular similarities and the fact that type A thymomas and MNTs often
203 co-occur in one tumor [3].

204 Type A thymomas and type A components of type AB thymoma rarely contained cell
205 clusters showing POU2F3/KIT double-expression, i.e., a feature of TSQCCs. This suggests a
206 previously unknown link between type A/AB thymomas with thymic SQCCs through the tuft
207 cell-like phenotype. This finding, together with the CD5 negativity of the POU2F3/KIT
208 double-positive clusters in type A/AB thymomas, supports the previously formulated
209 hypothesis [27] that POU2F3 may eventually drive KIT expression, and CD5 and KIT are
210 independently regulated in thymic SQCC. As to the rarity of POU2F3/KIT co-expressor type
211 A and AB thymomas, it is remarkable that this phenotype was associated with the rare
212 adenoid growth pattern [14, 26].

The reason why co-expression of POU2F3 and KIT is so rare in thymomas is unclear. As shown previously [27], co-expression of these markers is also rare in non-neoplastic tuft cells. Therefore, even if POU2F3 is indispensable for KIT expression in tuft cells or tuft cell-like cells, additional factors or cellular conditions might be necessary. The biggest difference between POU2F3(+)/KIT(+) cells and POU2F3(+)/KIT(-) cells in type A/AB thymomas seems to be the formation of tight clusters by the former. This is also a feature of POU2F3(+)/KIT(+) thymic carcinomas. Thus, signaling involving some adhesion molecule(s) might be related to KIT expression in TETs. A comprehensive comparison between POU2F3(+)/KIT(+) and POU2F3(+)/KIT(-) cells may be necessary to answer this question.

The cutoff value of the proportions of POU2F3-positive cells was changed here compared to the previous study [27] (from 40% to 10%) because the proportion of positive cells in thymic SQCC was generally lower in the current than previous study. Technical issues, such as the fixation status of TMA specimens, the different sizes of analyzed areas, or the protocol of IHC, might have caused this difference; however, it is also possible that the expression status of POU2F3 in TSQCCs is more heterogeneous in Japan, e.g., because ethnic effects may be operative.

Consistent with the significantly strong mRNA expression in thymic carcinoma [5, 6, 19, 27], positivity for L1CAM in IHC was also significantly higher in thymic carcinoma than

232 in all types of thymomas. However, unexpectedly, the correlation with POU2F3 expression
233 was low, and only two cases (2/17 [12%]) among the thymic carcinomas co-expressed both
234 markers on the protein level. Furthermore, no L1CAM-positive cells could be clearly
235 detected in the normal thymic medulla (not shown) despite its strong expression without
236 background staining in physiologically L1CAM-positive cells, such as renal tubular cells.
237 Therefore, although L1CAM is a useful diagnostic marker for TSQCC due to its specificity, it
238 is not certain that its expression is actually related to the tuft cell-like differentiation of
239 TSQCCs. Besides, because a subset of lung cancer, including SQCC, express this protein,
240 L1CAM-IHC is not helpful to distinguish thymic from lung cancers [24]. Nonetheless,
241 because L1CAM may be a biomarker for antiangiogenic interventions [12] that are a
242 cornerstone of recurrent TSQCC treatment [23] and may even be a suitable target for
243 improved therapy of metastatic and drug-resistant tumors [1, 2], the confirmation of L1CAM
244 protein expression in thymic carcinoma is potentially meaningful.

245 In summary, this study demonstrated the prevalence of POU2F3-positive cells in
246 TETs with different distribution patterns among histotypes. We found the following: 1)
247 thymic SQCC significantly frequently expresses POU2F3 across cohorts; 2) type A, AB, and
248 B1 thymomas often contain scattered POU2F3 (+) cells, while type B2 and B3 thymomas
249 generally do not; and 3) rare type A and AB thymomas contain POU2F3/KIT (+) clusters like
250 thymic SQCC. In particular, we would like to emphasize the presence of POU2F3 (+) cells in

type A thymoma, because POU2F3 can be an mTEC marker in the thymus and thymic tumors, and this finding seems consistent with the notion of medullary thymoma for this subtype [13].

Future studies should address the mechanism regulating the expression of POU2F3 and KIT in TETs. No adequate model mice for TETs exist, and long-term cultivation of representative TET cells *in vitro* is not yet possible. As with the challenge of establishing such tools for functional studies, the comprehensive expression profiling of neoplastic cells in TETs, ideally at the single-cell level, might hint at answers to the regulatory mechanism of POU2F3 and clarify in more detail the relationship among TET histotypes.

Declarations

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None declared

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Not applicable

Authors' contributions:

Drafting the manuscript and figures: YY. Acquisition and analysis of pathological data: YY, AS, MH, AY, and AM. Acquisition and analysis of clinical data: MH and HD. Correction and approval of the manuscript: all authors.

Code availability:

Not applicable

Ethics approval:

All experiments and procedures were approved by the Medical Ethics Committees of the Kyoto University Graduate School of Medicine and Kyoto University Hospital.

Consent to participate:

Not applicable

Consent for publication:

Not applicable

Figure legends

Figure 1. Presence of scattered POU2F3-positive cells in thymomas.

(a, b) Type A thymoma, (c, d) type AB thymoma, (e, f) type B1 thymoma showing rare POU2F3-positive cells. In type AB thymomas, POU2F3-positive cells occur in type A and type B-like components, while they are present in medullary islands and regions outside of them in type B1 thymoma (a, c, e: hematoxylin and eosin staining, b, d, f: immunohistochemistry)

Figure 2. Summary of POU2F3 and L1CAM positivity in thymic epithelial tumors.

A, type A (thymoma); AB, type AB; B1, type B1; B2, type B2; B3, type B3, MN, micronodular thymoma; Meta., metaplastic thymoma; TC, thymic carcinoma (16, squamous cell carcinoma; 1, mucoepidermoid carcinoma). MN cases were assessed with whole slide sections, while the other TETs were assessed with tissue microarrays (TMAs).

Figure 3. Type A thymoma with rare co-expression of POU2F3 and KIT.

A type A thymoma with adenoid pattern focally co-expresses POU2F3 (c) and KIT (d). The Ki-67 labeling index is low (< 1%) (e), and CD5 expression is not apparent (f).

Figure 4. L1CAM expression in thymic epithelial tumors.

(a, b) Thymic squamous cell carcinoma, (c, d) type A thymoma with POU2F3-positive cells. Thymic squamous cell carcinoma is positive for L1CAM. Type A thymoma does not express L1CAM despite the presence of POU2F3-positive cells.

Figure 5. Distribution of POU2F3 and KIT-positive cells in the thymus: A representative image.

A few POU2F3-positive thymic tuft cells are exclusively located in the medulla in normal human thymus. The epithelium of a multilocular thymic cyst can contain a small number of POU2F3-positive, presumably thymic tuft cells [22]. Thymic squamous cell carcinoma often contains POU2F3-positive cells [27]. Type A, AB, and B1 thymomas sometimes contain scattered POU2F3-positive cells. The distribution is generally heterogeneous irrespective of histotypes. They are located in type A and type B-like components, and MIs in type AB thymoma, and type A component and MIs are generally easier areas to detect these cells. In type B1 thymomas, POU2F3(+) cells are generally observed in MIs, although these cells are located outside of MIs in rare cases. Type B2 and B3 thymomas generally lack POU2F3-positive cells. Type A/AB thymoma, possibly with adenoid pattern, rarely contains POU2F3 aggregations, which co-express KIT.

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Figure 1

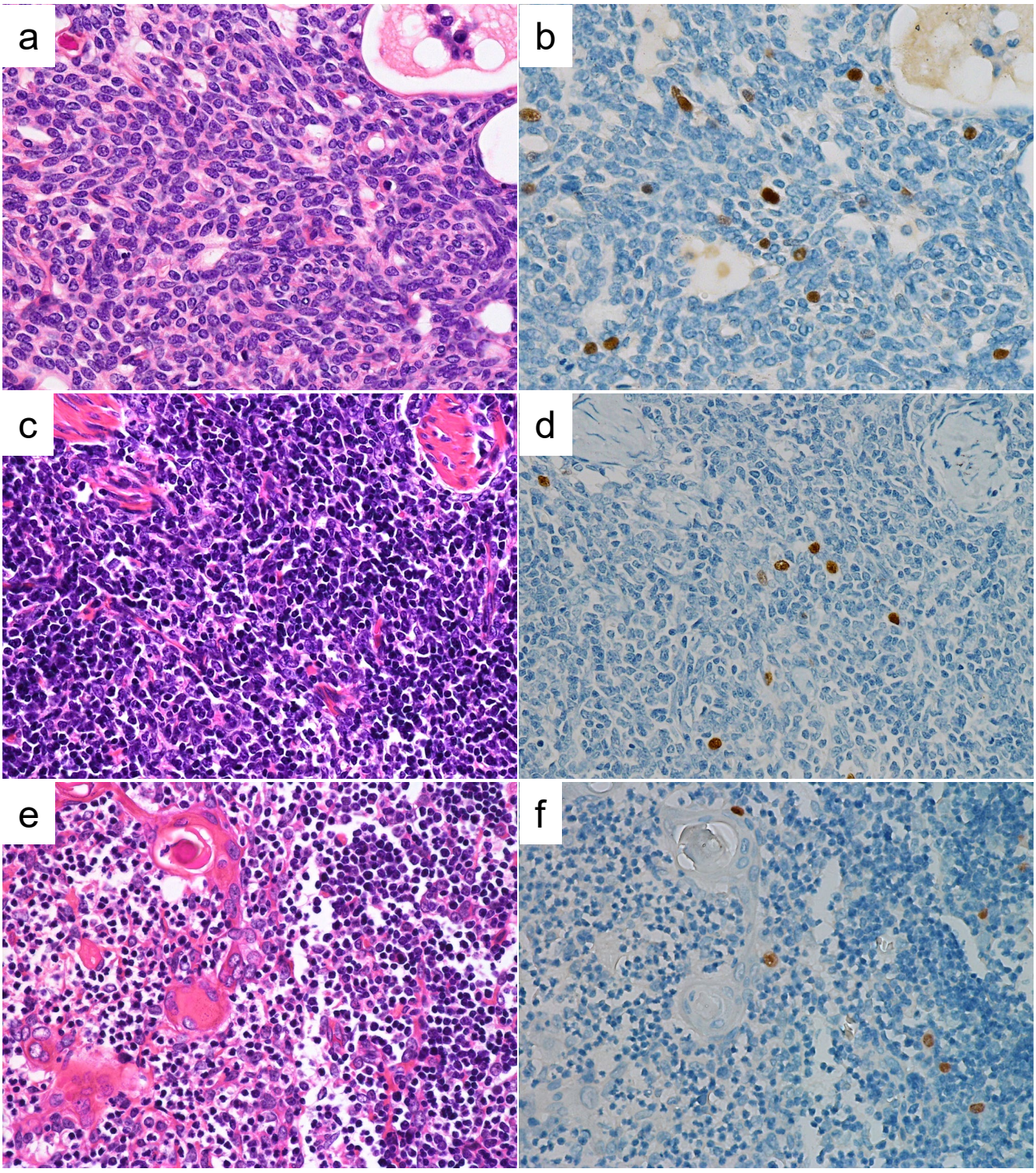


Figure 2

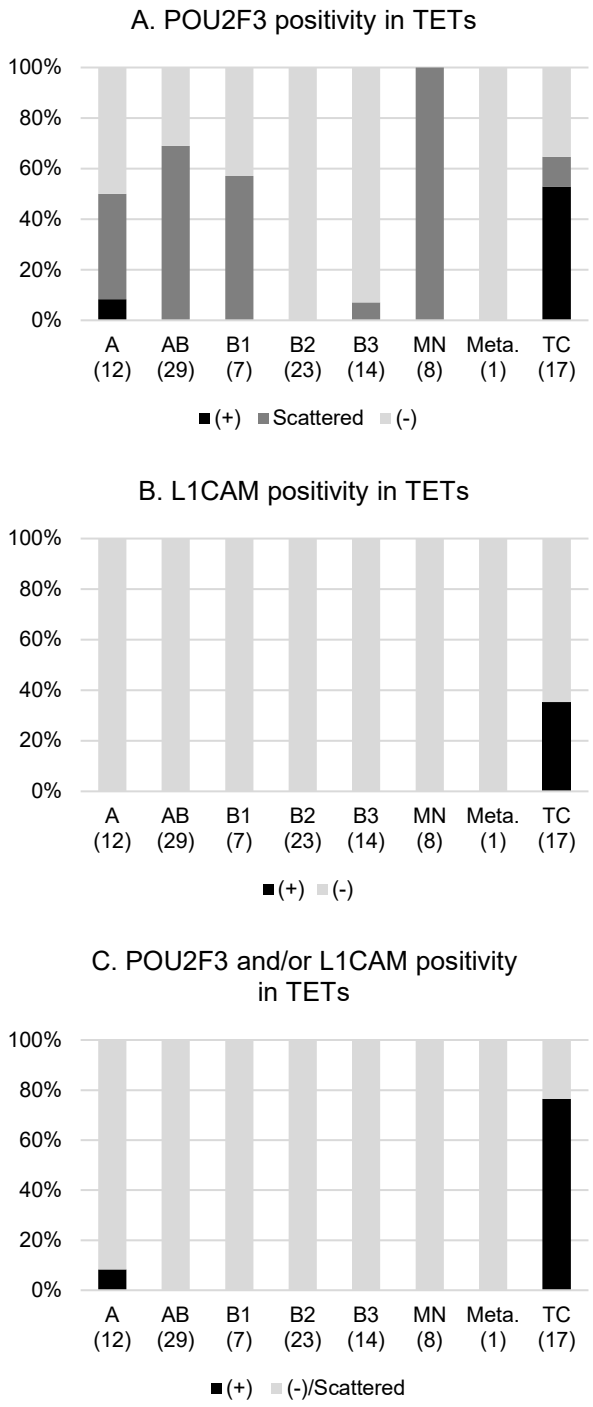
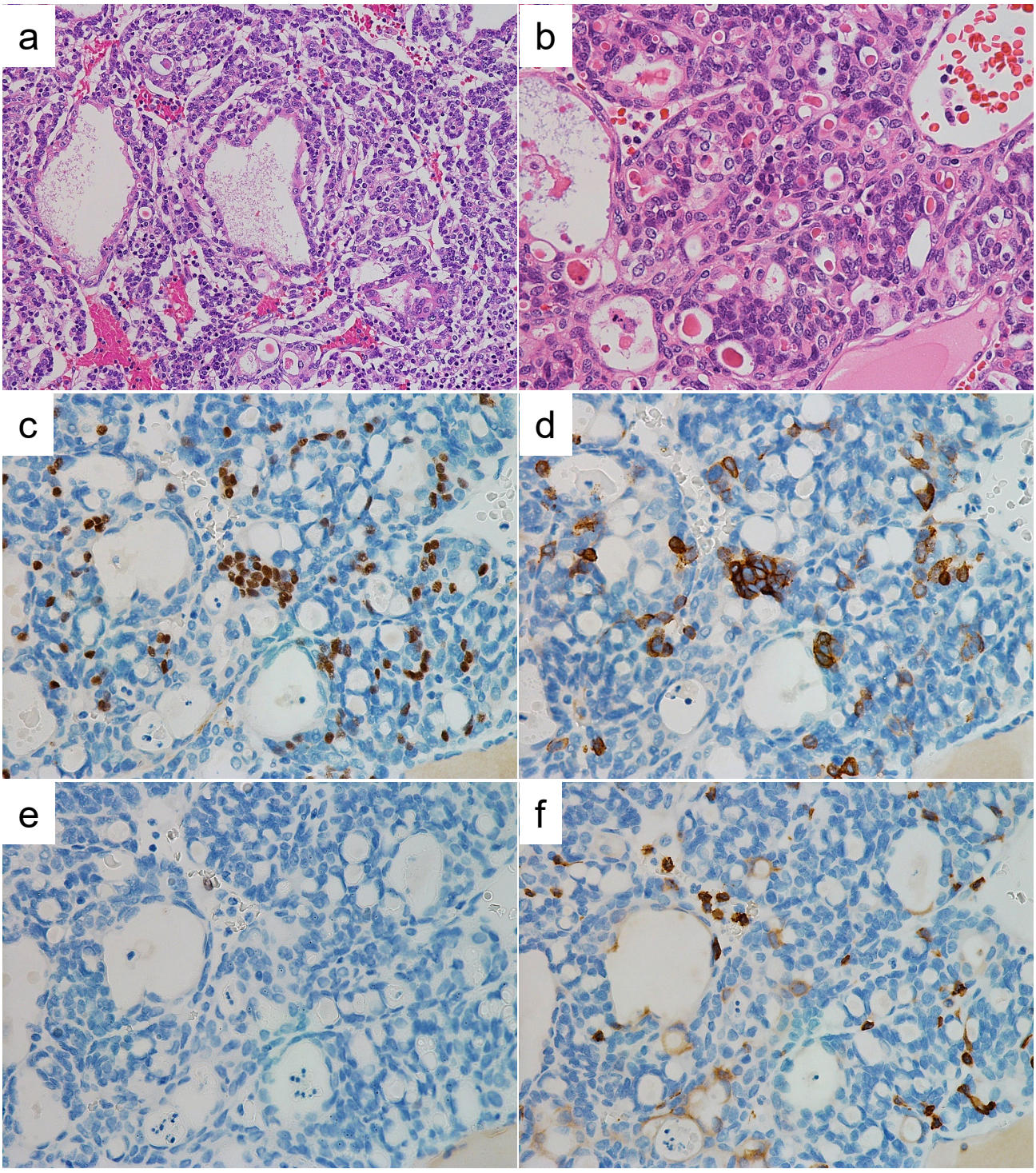

A. POU2F3 positivity in TETs
100%
80%
60%
40%
20%
0%
A (12)
AB (29)
B1 (7)
B2 (23)
B3 (14)
MN (8)
Meta. (1)
TC (17)
(+)
Scattered
(-)
B. L1CAM positivity in TETs
100%
80%
60%
40%
20%
0%
A (12)
AB (29)
B1 (7)
B2 (23)
B3 (14)
MN (8)
Meta. (1)
TC (17)
(+)
(-)
C. POU2F3 and/or L1CAM positivity in TETs
100%
80%
60%
40%
20%
0%
A (12)
AB (29)
B1 (7)
B2 (23)
B3 (14)
MN (8)
Meta. (1)
TC (17)
(+)
(-)/Scattered

Figure 3



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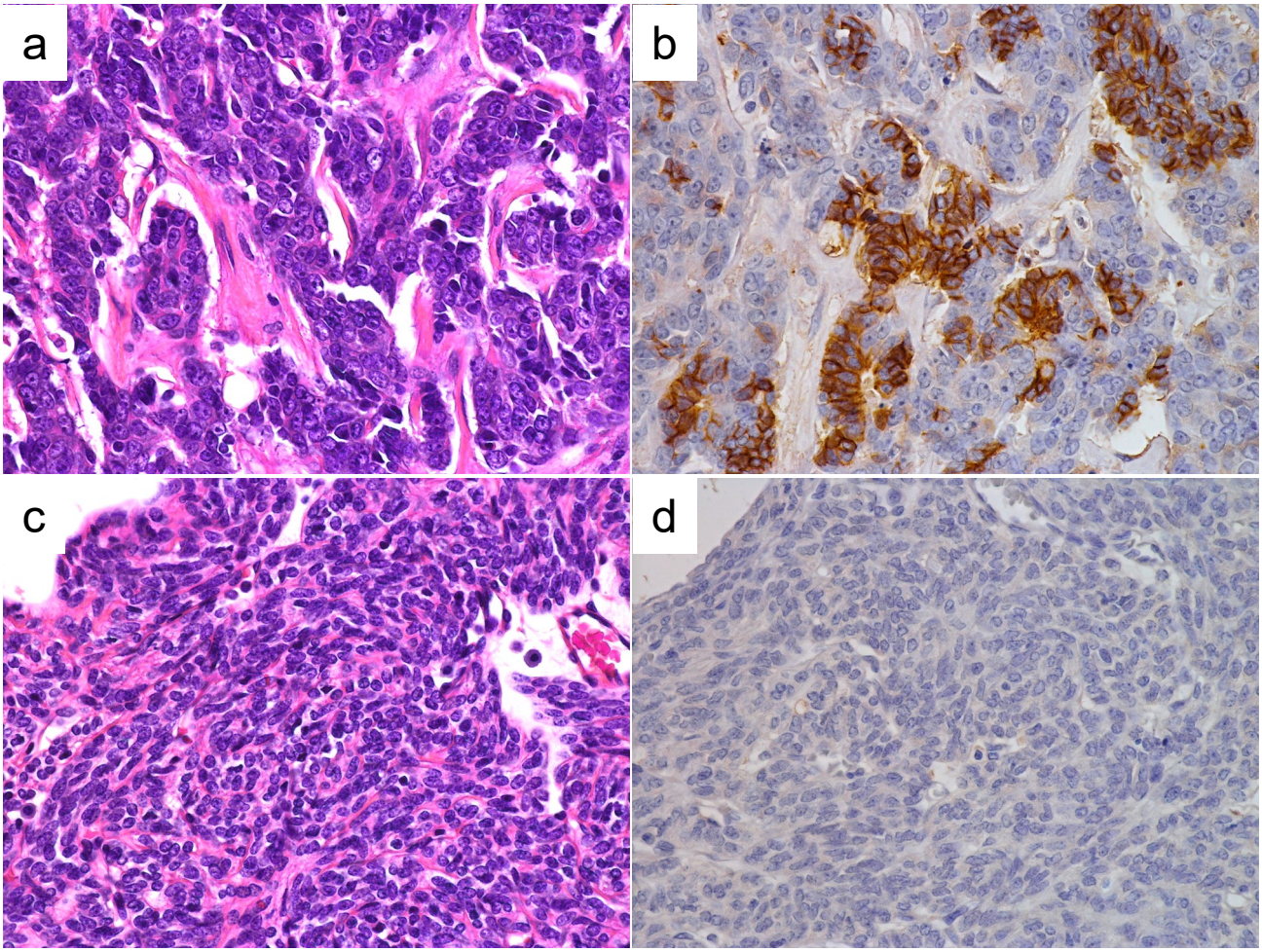

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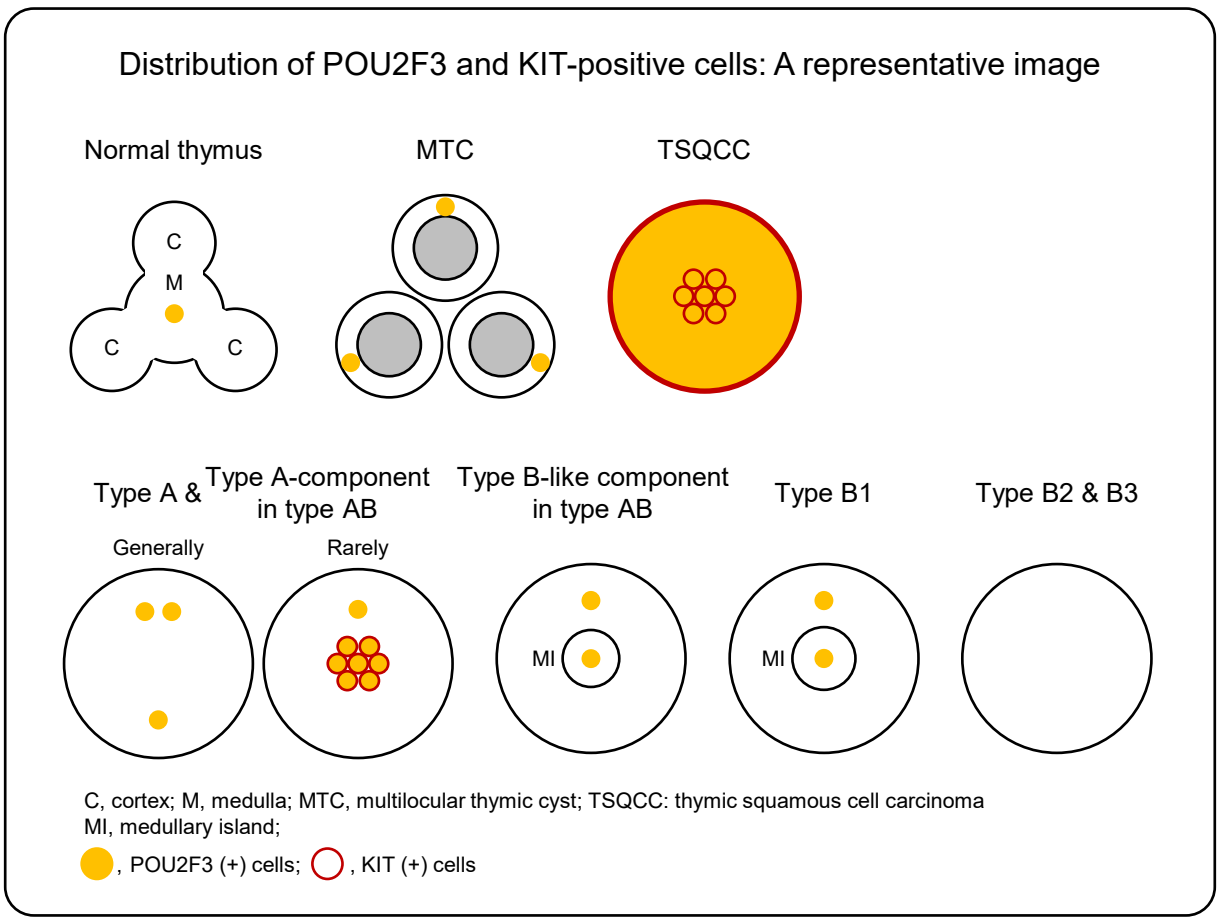

Distribution of POU2F3 and KIT-positive cells: A representative image
Normal thymus
MTC
TSQCC
C
M
C
C
Type A &
Type A-component in type AB
Type B-like component in type AB
Type B1
Type B2 & B3
Generally
Rarely
MI
MI
C, cortex; M, medulla; MTC, multilocular thymic cyst; TSQCC: thymic squamous cell carcinoma
MI, medullary island;
, POU2F3 (+) cells; , KIT (+) cells

Figure S1

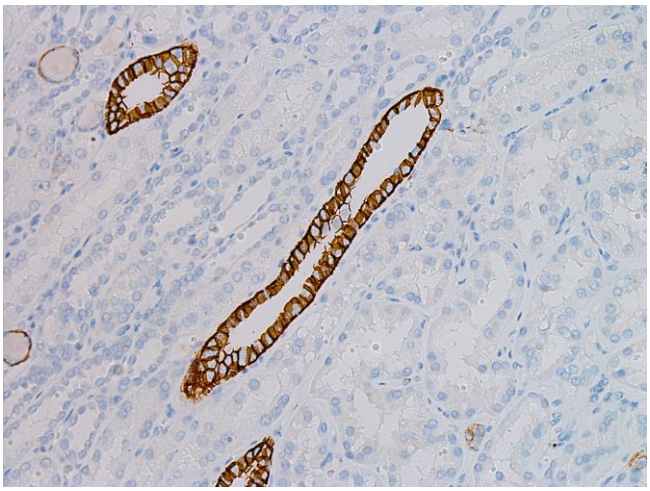


Figure S2

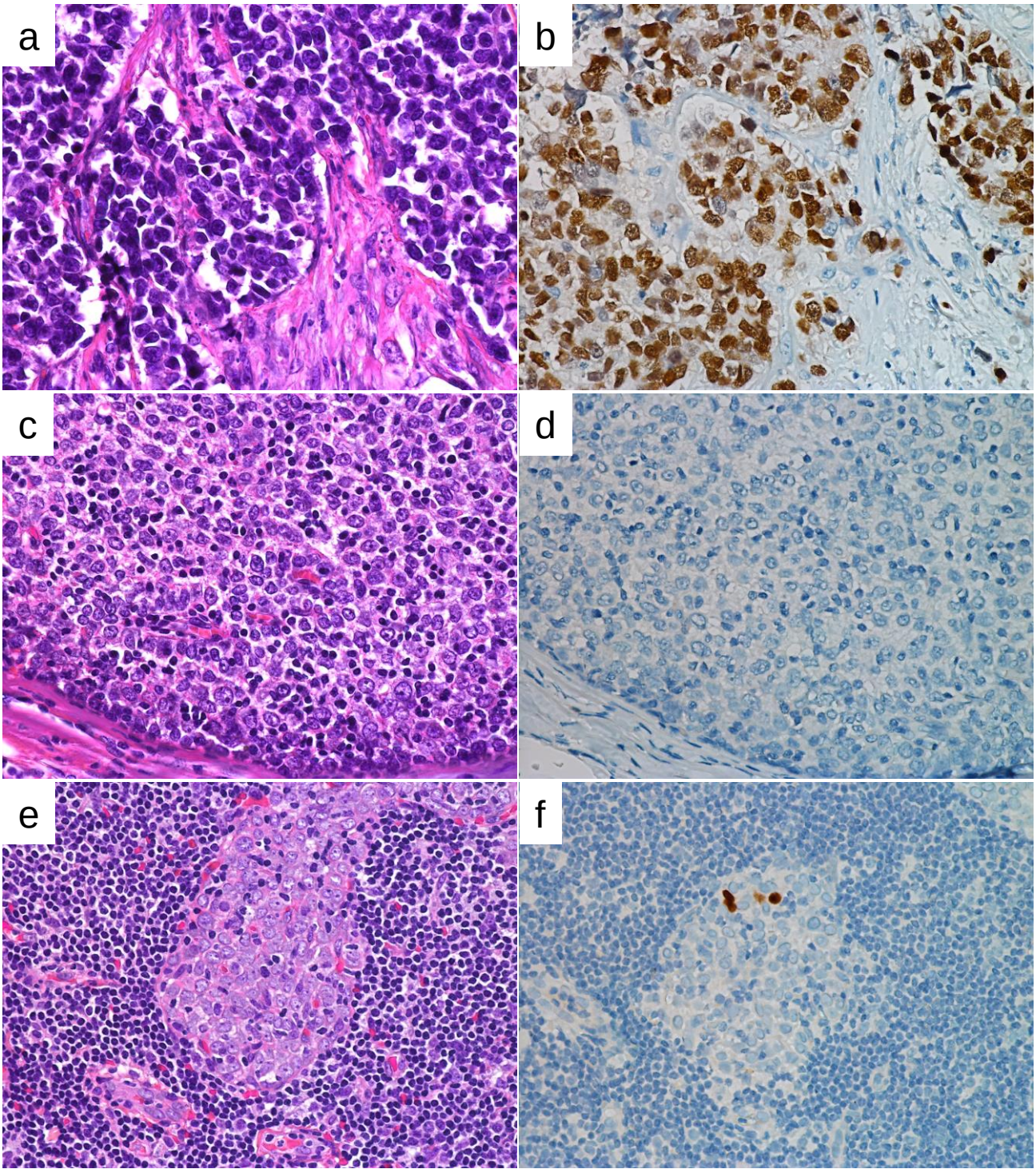


Figure S3

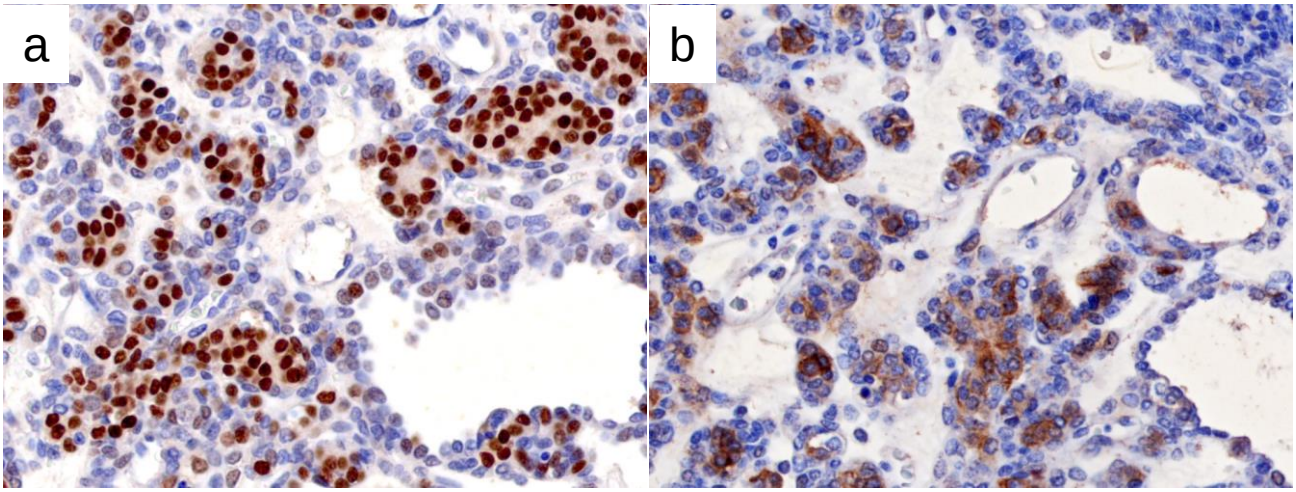
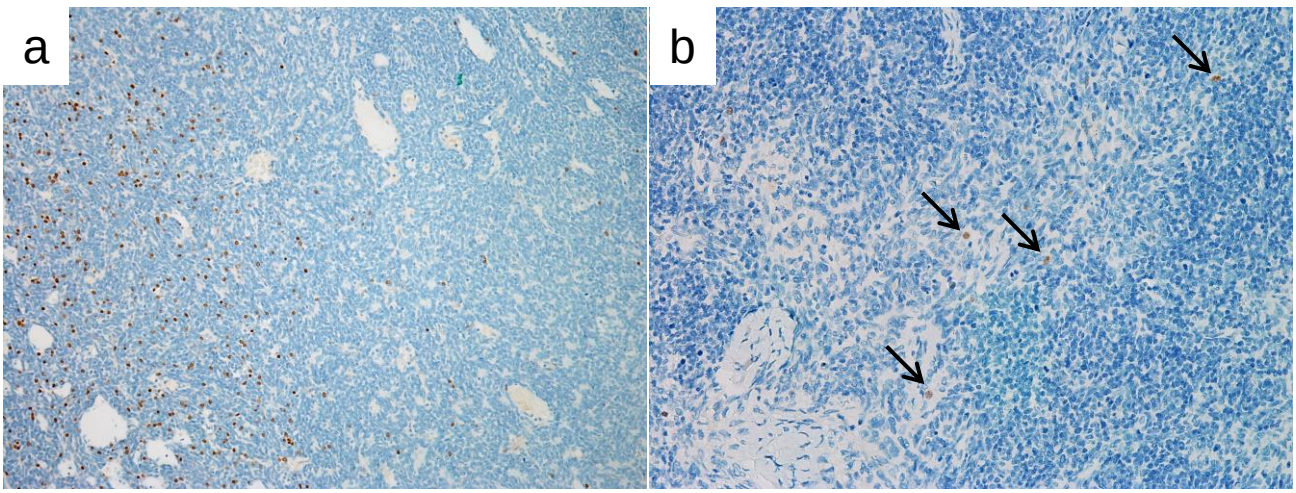


Figure S4



Supplementary figure legends

Figure S1. L1CAM expression in renal tubule cells.

Figure S2. Expression pattern of POU2F3 in thymic squamous cell carcinoma, type B3 thymoma, and micronodular thymoma with lymphoid stroma.

(a, b) Thymic squamous cell carcinoma, (c, d) type B3 thymoma, (e, f) micronocular thymoma with lymphoid stroma. Thymic squamous cell carcinoma clearly expresses POU2F3. Type B3 thymoma lacks POU2F3 expression. Micronodular thymoma contains scattered POU2F3-positive cells (a, c, e: hematoxylin and eosin staining. b, d, f: immunohistochemistry)

Figure S3. Type AB thymoma with rare co-expression of POU2F3 and KIT

A type AB thymoma with adenoid pattern in the type A component focally co-expresses POU2F3 (a) and KIT (b).

Figure S4. Spatial distribution of POU2F3(+) cells in thymoma.

The distribution of POU2F3(+) cells is generally heterogeneous irrespective of histotypes (a, type A thymoma). POU2F3(+) cells (arrows) tended to be located in type A compared with type B-like components in type AB thymoma (b).