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ORIGINAL ARTICLE

Identification of invasive subpopulations using spatial transcriptome analysis in thyroid follicular tumors

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Background: Follicular tumors include follicular thyroid adenomas and carcinomas; however, it is difficult to distinguish between the two when the cytology or biopsy material is obtained from a portion of the tumor. The presence or absence of invasion in the resected material is used to differentiate between adenomas and carcinomas, which often results in the unnecessary removal of the adenomas. If nodules that may be follicular thyroid carcinomas are identified preoperatively, active surveillance of other nodules as adenomas is possible, which reduces the risk of surgical complications and the expenses incurred during medical treatment. Therefore, we aimed to identify biomarkers in the invasive subpopulation of follicular tumor cells. **Methods:** We performed a spatial transcriptome analysis of a case of follicular thyroid carcinoma and examined the dynamics of CD74 expression in 36 cases. **Results:** We identified a subpopulation in a region close to the invasive area, and this subpopulation expressed high levels of CD74. Immunohistochemically, CD74 was highly expressed in the invasive and peripheral areas of the tumor. **Conclusions:** Although high CD74 expression has been reported in papillary and anaplastic thyroid carcinomas, it has not been analyzed in follicular thyroid carcinomas. Furthermore, the heterogeneity of CD74 expression in thyroid tumors has not yet been reported. The CD74-positive subpopulation identified in this study may be useful in predicting invasion of follicular thyroid carcinomas.

Key Words: Thyroid; Follicular carcinoma; Spatial transcriptome analysis; CD74; Capsular invasion

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Follicular tumors include follicular thyroid adenomas and carcinomas, which are differentiated by the presence of capsular or vascular invasion in the resected materials or metastasis [1]. Owing to the absence of morphological differences between the tumor cells, fine-needle aspiration cytology, in which the contents of the nodule are sampled, and tissue biopsy, in which a portion of the tumor is sampled, cannot differentiate between them preoperatively [2]. Hence, many adenomas are unnecessarily resected [3,4]. If potential follicular thyroid carcinoma nodules are identified preoperatively, active surveillance of adenomas is possible, which could reduce the risk of surgical complications and decrease the cost of medical treatment.

Many studies have reported methods to distinguish follicular thyroid adenomas from carcinomas [5-8]. Some of them require special equipment and techniques for analysis [5,6]. Markers used in immunohistochemistry may be helpful to distinguish between the two using simple methods. Several studies have suggested markers for this purpose; however, no definitive marker worthy of clinical application has yet been found [7-12]. Spatial transcriptome analysis technology for formalin-fixed paraffin-embedded (FFPE) specimens has advanced considerably, and tumor heterogeneity can be detected [13,14]. If invasive tumor subpopulations can be detected by spatial transcriptome analysis of FFPE specimens of follicular thyroid carcinomas, new markers for assessing the invasiveness of follicular thyroid carcinoma may be identified. The eligibility of the target tumors for resection can

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be determined during tissue biopsy and cytology. In this study, we aimed to identify tumor subpopulations composing invasive areas using spatial transcriptome analysis of FFPE specimens containing the invasive area of follicular thyroid carcinoma and to perform immunohistochemical studies based on these results.

MATERIALS AND METHODS

Patients

Between May 2016 and December 2022, 283 follicular thyroid carcinoma nodules were resected at the Kuma Hospital in Japan. Of these, 36 nodules (minimally invasive [n = 18], encapsulated angioinvasive [n = 8], widely invasive [n = 10]) were extracted after excluding those with papillary-like nuclear features, poorly differentiated carcinoma components, and very small invasive areas. A nodule with an invasive area within a 5-mm-diameter circle was subjected to spatial transcriptome analysis, and all cases were subjected to immunohistochemical staining. The clinicopathological features of the enrolled 36 cases are shown in Table 1.

Spatial transcriptome analysis

We performed spatial transcriptome analysis using Visium CytAssist Spatial Gene Expression for FFPE (10x Genomics, Pleasanton, CA, USA). In this analysis, the whole RNA transcriptome of cells in each spot of the specialized slides was obtained from FFPE tissue sections. Each spot contained approximately 10–20 cells, and the RNA transcriptome of these cells revealed the features of RNA expression in each spot. The tissues were sectioned as described in the Visium CytAssist Spatial Gene Expression for FFPE Tissue Preparation Guide (CG000518). Sections were stained with hematoxylin and eosin (H&E), imaged, and de-cover-slipped, followed by H&E de-staining and de-crosslinking. Glass slides with tissue sections were processed using a Visium CytAssist instrument to transfer analytes to a Visium CytAssist Spatial Gene Expression slide with a 0.42 cm^2 capture area. Probe extension and library construction steps followed the standard Visium for the FFPE workflow. Libraries were sequenced using a DNBSEQ-G400 sequencer (BGI) (read 1: 28 bp, read 2: 100 bp). Spatial data were pre-processed and aligned using 10x Genomics Space Ranger v1.3.0 with the reference human genome GRCh38 (refdata-gex-GRCh38-2020-A) to generate raw unique molecular identifier count spot matrices. Dimensionality reduction was performed using classical principal component analysis integrated uniform manifold approximation and projection (UMAP) [15]. Unsupervised clustering of the data spots was performed using Louvain clustering [16] integrated into the BioTuring Lens with a resolution of 0.1. Further downstream analysis and gene expression visualization were conducted using the BBrowser (BioTuring, San Digo, CA, USA).

CD74 immunohistochemical analysis

Immunohistochemical staining was performed using 3-μm-thick FFPE specimens. Anti-CD74 (1:100, LN2, ab9514, Abcam, Cambridge, UK) and anti–thyroid transcription factor-1 (TTF-1; 1:100, SP141, ab227652, Abcam) were used as primary antibodies. Staining was performed using a Dako Autostainer Link 48+ (Dako, Carpinteria, CA, USA), according to the manufacturer's recommendations. The expression of CD74 was assessed using a visual grading system based on staining intensity under light microscopy. High intensity (++), low intensity (+), and no signal (–) were defined as strong, weak, and no staining, respectively. The histological index was calculated using the following formula:

Histological index = 2 × (% cells of high intensity) + 1 × (% cells of low intensity).

The invasive area was defined as a tumor lesion invading or over the capsule or an angioinvasive lesion. The peripheral area of the tumor was defined as the tumor lesion within 1 mm of the tumor border. The tumor central area was defined as the tumor lesion > 1 mm from the border. One pathology assistant (A.S) and one pathologist (E.M) scored the data independently.

RESULTS

Spatial transcriptome analysis

Among the 36 cases of follicular thyroid carcinoma, a nodule with an invasive area within a circle of 5 mm diameter was subjected to spatial transcriptome analysis (Fig. 1A). As tissue samples in the circle could be examined in the spatial transcriptome

Table 1. Clinicopathological features of enrolled cases

	Minimally invasive	Encapsulated angioinvasive	Widely invasive	Total
No.	18	8	10	36
Sex (male:female)	3:15	4:4	2:8	9:27
Age (yr), mean (range)	49.1 (27–83)	43.5 (11–68)	47.3 (12–69)	47.4 (11–83)

analysis, we selected a case in which the invasive and non-invasive areas were involved in the circle. A minimally invasive case was selected for spatial transcriptome analysis. The invasive, peripheral, and central areas were shown in Fig. 1B. Louvain clustering with a resolution of 0.1 showed three clusters, and the H&E staining image merged with the three clusters (Fig. 1C). Dimensionality reduction using classical principal component analysis integrated with UMAP showed that the features of cluster 1 were distinct from those of clusters 2 and 3 (Fig. 1D). Cluster 1 was detected in the invasive area, where clusters 2 or 3 were not found (Fig. 1C, asterisk portion). The BioTuring Lens showed 10 highly expressed genes in each cluster (Table 2). CD74 was highly expressed in cluster 1 but not in clusters 2 or 3. We examined the expression of CD74 with immunohistochemistry in all 36 cases of follicular thyroid carcinoma.

CD74 immunohistochemical analyses

Immunohistochemically, the CD74 staining intensity was heterogeneous. The intensity increased in the invasive and peripheral areas of the tumors (Fig. 2A, B). Since CD74 is expressed in macrophages, double staining for CD74 and TTF-1 was performed to confirm that CD74-positive cells were follicular thyroid carcinoma cells. CD74-positive cells in the invasive and peripheral areas were TTF-1 positive, indicating that CD74

Table 2. Top 10 highly expressed genes in each cluster

Cluster 1	Cluster 2	Cluster 3
CD74	COL9A3	COL9A3
IGHG1	SFRP1	APLP2
IGHG3	APLP2	MT1G
IGHM	CD24	CD24
B2M	GPX3	SFRP1
IGHA1	MT1G	SLC26A7
COL1A1	PDCD4	PDCD4
COL3A1	SLC26A7	GPX3
ACTB	IGFG1	FCGBP
FOS	B2M	APP

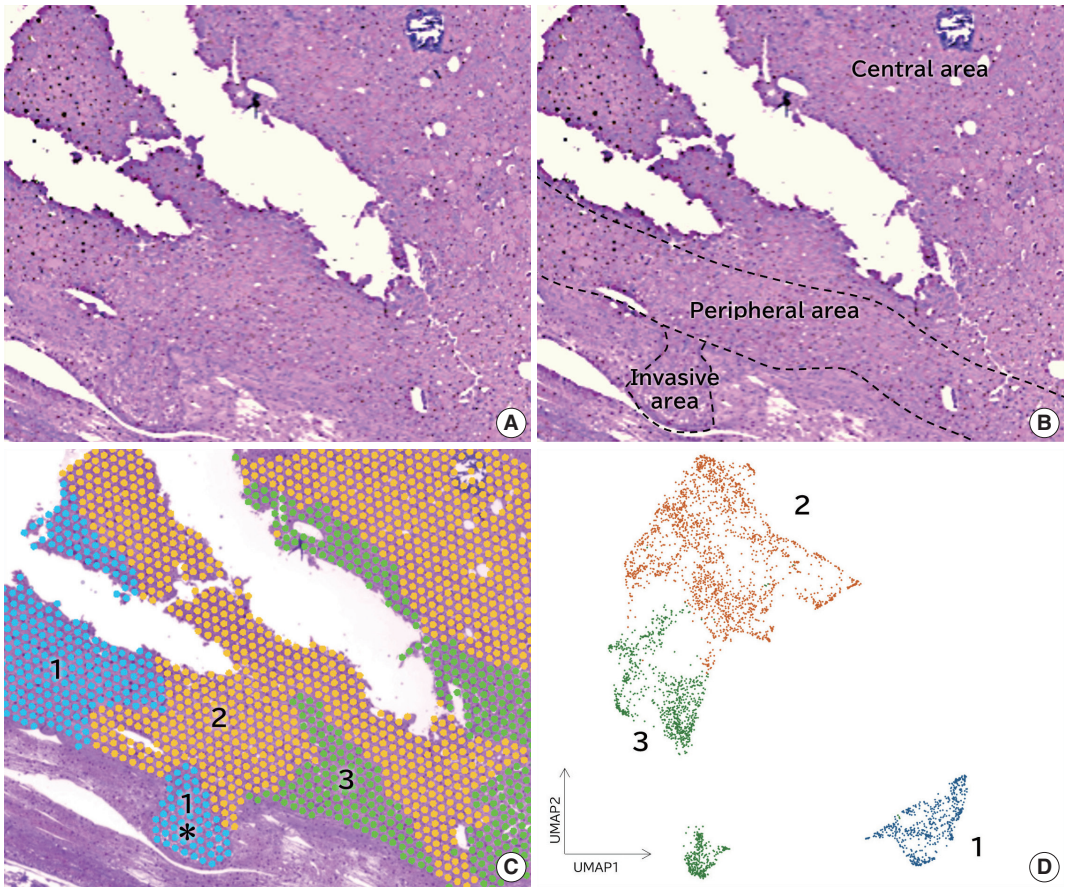

Fig. 1. Spatial transcriptomic analysis of follicular thyroid carcinoma with minimally invasive area. (A) Hematoxylin and eosin staining of examined case. (B) The invasive, peripheral, and central areas are shown. (C) Three clusters are obtained by Louvain clustering with a resolution of 0.1. Invasive area is shown with the asterisk. (D) Features of three clusters obtained by dimensionality reduction through classical principal component analysis integrated uniform manifold approximation and projection (UMAP).

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positivity was detected in tumor cells located in the invasive and peripheral areas (Fig. 2C, D).

We evaluated the CD74 staining intensity in follicular thyroid carcinoma cells using a histological index (Fig. 3A). The histological index was 98.1 ± 50.6 (mean ± standard deviation) in invasive areas, 96.0 ± 48.5 in peripheral areas, and 65.8 ± 36.2 in central areas (Fig. 3B, C). Significant differences were detected between the invasive and central areas and between the peripheral and central areas ($p < .01$).

DISCUSSION

Follicular thyroid adenomas and carcinomas are collectively treated as follicular tumors. They are difficult to differentiate using fine-needle aspiration cytology [2], and the clinical management is not differentiated [3]. However, because follicular thyroid adenomas, which comprise the majority of follicular tumors, are benign, unnecessary resection is often performed in many cases. Several studies have proposed the use of various markers for distinguishing between follicular thyroid adenomas and carcinomas. Sato et al. [5] reported the utility of p53-binding protein 1 (53BP1) expression in nuclear foci, a marker reflecting the DNA damage response, to distinguish follicular thyroid carcinomas from adenomas. 53BP1 showed high sensitivity (89.3%) and specificity (83.3%), although the interpretation of results required advanced techniques. Suzuki et al. [6] attempted to differentiate between the two tumor types by determining the cell proliferation index reflecting increased nuclear DNA levels using a special flow cytometry device, LC-1000 (Sysmex Corporation, Tokyo, Japan). This method automatically calculates the index; however, a huge initial investment is required. An easier method to incorporate is immunohistochemistry, and antibodies such as ki-67, bax, secreted protein acidic and rich in cysteine, HBME1, Rac1, Galectin-3, and CD61 have been investigated, but none of them is currently in clinical use [7-12].

RNA sequences of microdissected samples are useful for identifying molecular markers; however, they only provide information on the bulk expression of tumor cells in the microdissected area. The most precise investigation of RNA expression is a single-cell RNA sequence; however, this method loses the spatial

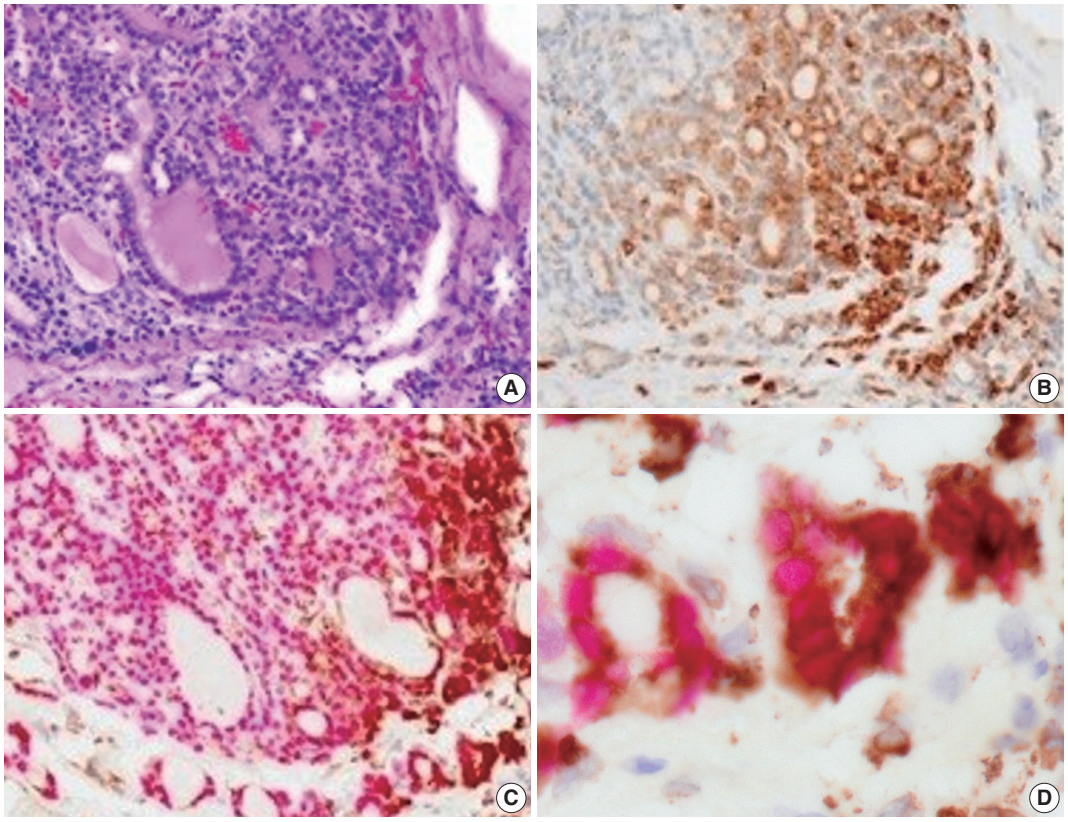

Fig. 2. Immunohistochemical analyses of CD74 in follicular thyroid carcinoma. (A) Hematoxylin and eosin staining. (B) Immunohistochemical staining using anti-CD74 antibody. (C) Double immunohistochemical staining using anti-CD74 (brown) and anti–thyroid transcription factor-1 (red) antibodies. (D) High power field of (C).

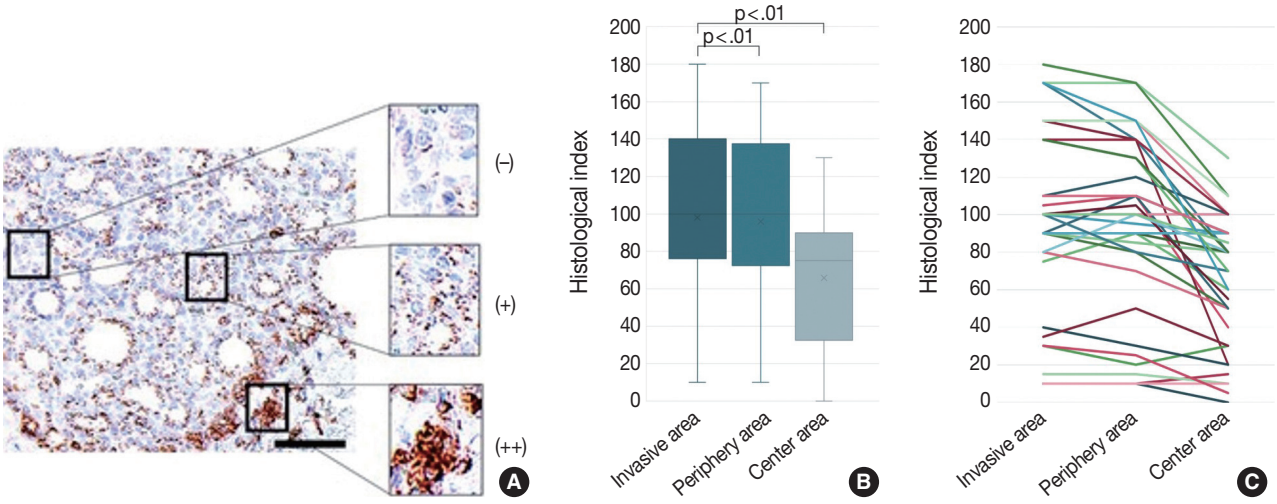

Fig. 3. Evaluation of CD74 staining intensity with histological index. (A) Typical image of CD74 immunohistochemical staining. Staining intensity was divided into three categories; high intensity (++), low intensity (+), and no signal (–) defined as strong, weak, and no staining, respectively. (B) Box plot of CD74 staining intensity in invasive, peripheral, and central areas. Significant differences were detected between the invasive and central areas, and between peripheral and central areas. (C) Line graph of CD74 staining intensity in each case.

information of each individual cell. Spatial transcriptome analysis is a new technique that enables whole-transcriptome analysis without microdissection of FFPE specimens while maintaining morphological information; it has been investigated in a variety of tissues [13,14]. In this study, we performed spatial transcriptome analysis using follicular thyroid carcinoma and identified a subpopulation in the invasive area that was found to express high levels of CD74.

CD74 is known to play an important role in antigen presentation by mediating the construction of major histocompatibility complex class II complexes and intracellular trafficking [17]. CD74 has also been reported to be upregulated in malignant tumors and involved in increased growth and metastatic potential [18-28]. In a study on urothelial bladder carcinomas by Choi et al. [18], urothelial bladder carcinomas with high CD74 expression were characterized by older age, high World Health Organization grade, and advanced stages of TNM classification. In a study on gastrointestinal carcinomas, Gold et al. [26] found that the expression of CD74 in gastrointestinal carcinomas was significantly greater than that in their normal tissue counterparts ($p < .001$ or lower). With regard to thyroid carcinomas, Varinelli et al. [27] and Cheng et al. [28] reported that the CD74 axis plays an important role in the biology of aggressive cases in papillary and anaplastic thyroid carcinomas. In these two studies [27,28], CD74 staining was detected in all tumor cells of aggressive cases, and no heterogeneous staining was reported. To our knowledge, the present study is the first report of CD74 staining in follicular thyroid carcinomas and the heterogeneous staining intensity of CD74 was detected for the first time, suggesting the presence of an invasive subpopulation of follicular tumor cells. In a study by Cheng et al. [28], treatment with anti-CD74 antibody in papillary thyroid carcinoma cell lines inhibited cell growth, colony formation, cell migration and invasion, and vascular endothelial growth factor secretion. Our results suggested that CD74 might play a role in cell invasion and might be a novel therapeutic target for follicular thyroid carcinomas, such as papillary thyroid carcinoma.

Our results demonstrated a significantly higher staining score in the invasive area than in the central area ($p < .01$), suggesting that the CD74-positive subpopulation may be used to predict the invasion of follicular tumors. Although there was no significant difference between the invasive and peripheral areas just below the capsule ($p = .429$), Gold et al. [26] stated that precursor lesions might express the same or higher CD74 levels as the respective cancers, as the activation of survival pathways was particularly important in the early stages of tumorigenesis. It was possible that the positive cells observed immediately below the capsule in our study were near invasion. Although this study was performed on resected material, we expect that immunocytochemical staining for CD74 will be performed in the future on nodules suspected of having follicular tumors on cytological examination or biopsy tissue specimens to determine the possibility of invasion before surgery, which will contribute to reducing unnecessary resection of nodules with a low possibility of invasion.

In this study, we only included follicular thyroid carcinomas with a clear invasion. Future studies are required to examine CD74

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immunostaining in patients with follicular tumors of uncertain malignant potential [1] with unclear histological status to identify whether they are follicular thyroid adenomas or carcinomas and prospectively observe their prognosis. The correlation between the staining results and prognosis provided a better confirmation of the relationship between CD74 immunostaining and invasion.

Ethics Statement

The study protocol was reviewed and approved by the Institutional Review Board of Kuma Hospital (no. 20230112-2, 12/01/2023) and Osaka University Clinical Research Review Committee (no. 22425, 17/02/2023). This study complied with the 1964 Declaration of Helsinki and its later amendments. All study participants provided informed consent.

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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