Original Article

Expressions of D2-40, CK19, galectin-3, VEGF and EGFR in papillary thyroid carcinoma

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Objective: To investigate the expressions of D2-40, CK19, galectin-3, VEGF, and EGFR in papillary thyroid carcinoma and their clinical significances.

Methods: The expressions of D2-40, CK19, galectin-3, VEGF, and EGFR in 38 cases of papillary thyroid carcinoma and 12 cases of thyroid papillary hyperplasia were detected by immunohistochemical staining.

Results: The positive expression rates of D2-40, CK19, Galectin-3, VEGF and EGFR in patients with papillary thyroid carcinoma were all significantly higher than those in patients with thyroid papillary hyperplasia (all $P<0.05$). The expressions of D2-40, VEGF, and EGFR in papillary thyroid carcinoma with lymph node metastasis were significantly higher than those without lymph node metastasis ($P<0.05$). The expressions of GK19 and galectin-3 showed no significant differences between the papillary thyroid carcinomas with and without lymph node metastasis ($P>0.05$).

Conclusions: The detection of D2-40, CK19, galectin-3, VEGF, and EGFR is helpful for the diagnosis and differential diagnosis of papillary thyroid carcinoma and thyroid papillary hyperplasia.

Keywords: Thyroid neoplasms; papillary carcinoma; D2-40; CK19; galectin-3; VEGF; EGFR; immunohistochemis

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Introduction

Thyroid carcinoma accounts for 1% in systemic malignancies tumors (1), and they cause more deaths than all endocrine organs cancers. Early diagnosis and prompt treatment of thyroid cancer may maximize the survival rate and prolong survival time of patients. Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancies, approximately 75-85% of thyroid cancers are papillary carcinoma (1). It originates from the thyroid follicle cells. However, distinguishing papillary thyroid carcinoma from thyroid papillary hyperplasia is extremely challenging due to tumor heterogeneity. On occasion cases of papillary thyroid hyperplasia, in particular solitary nodules with papillary change, can simulate papillary thyroid carcinoma and cause a diagnostic dilemma (2). In addition, PTC gives frequently rise to nodal metastases via lymphatic vessels. Metastases occur in 20% of patients. Most commonly, they metastasize to the lungs, bones, liver and the brain.

In the recent years, the development of PTC is influenced by many factors including genetic alterations, growth factors, and physical agents such as radiation. Useful prognostic factors are needed for determining biologic behavior, providing an initial assessment. And a large number of molecular alterations have been used in differential diagnosis of papillary thyroid carcinoma. These biomarkers, such as D2-40, cytokeratin 19 (CK19), galectin-3, Vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR) have been translated into clinical practice which offered significant improvement in the preoperative diagnosis of thyroid cancer (3-9).

D2-40 is a newly identified monoclonal antibody that

specifically binds to a 40,000 asialoglycoprotein M2A (tumor embryonic antigen). M2A is often used to mark lymphatic vessels distinguishably from vascular endothelial cells in laboratory settings as it is expressed in lymphatic endothelial cells instead of the vascular endothelium, so it is considered the most specific lymphatic marker (10). CK19 is a low molecular weight cytokeratin, and there have been several reports on its role in the diagnosis and differential diagnosis of thyroid benign and malignant lesions (11,12). Some investigators (13,14) also noted focal expression of CK19 in benign thyroid lesions. Galectin-3, a member of the B galactosil binding lectin family, for which normal functions include cell-cell regulation, growth, and differentiation in some studies. Finding that the sensitivity and specificity of galectin-3 were 93% and 100%, respectively, for papillary thyroid carcinoma, Finley (15) suggested that galectin-3 could contribute to the differentiation between benign and malignant thyroid papillary hyperplasia. His view was supported by Cvejić (16). VEGF is a main promoter of endothelial growth and migration, many studies have shown a correlation between expression of it and prognosis in several cancers, including well-differentiated thyroid cancer (7,17). Several aspects of the relationship between thyroid cancer and VEGF expression have been studied (18), including prognosis and the presence of metastasis. EGFR is a member of the Erb family of receptors which is abnormally activated in many epithelial tumors and is one of the receptors often found up-regulated in human carcinomas and often related to poor prognosis or advanced pathological stages (19). Yeh *et al*. have shown that EGFR is involved in cancer cell invasion (20).

However, few efforts have been made for a systematic detection of multiple antibodies. In this study, the purpose was to assess the significances of D2-40, CK19, galectin-3, VEGF and EGFR expression in the diagnosis and differential diagnosis of papillary thyroid carcinoma.

Materials and methods

Specimens and general information

The surgical specimens were collected from 38 patients with papillary thyroid carcinoma and 12 patients with benign thyroid disease with papillary hyperplasia in the Department of Pathology, Jilin City Central Hospital from January 2008 to December 2009. All of the thyroid lesion specimens were pathologically confirmed and classified according to the WHO classification criteria for thyroid tumors. The subjects were divided into: (I) papillary carcinoma group: 12 men and 26 women, at the age from 31 to 64 years (mean 49.7); (II) papillary hyperplasia group: 3 men and 9 women, at the age from 25 to 54 years (mean 42.4 years). All patients were treatment-naive and had complete clinical and pathological data. Each section was reviewed by two pathologists at the level of deputy director or above.

Experimental methods

Immunohistochemical method

Specimens were fixed in 10% neutral buffered formalin, processed conventionally, embedded in paraffin, cut into 4μm sections and stained with hematoxylin and eosin (HE). The immunohistochemical SP assay was conudcted following the instructions in the kit insert. Reagents used in this study included mouse anti-human D2-40, CK19, galectin-3, VEGF and EGFR (ready-for-use agents). All of the reagents, SP broad-spectrum ultra-sensitive kits and diamino benzidine (DAB) staining kits were purchased from Fuzhou Maixin Company. Phosphate buffered saline (PBS) was used as negative control in place of the primary antibody.

Determination of results

The galectin-3 positive signal was recognized when brownish-yellow granules were present in the cytoplasm, CK19-positive signal when in both the cytoplasm and cell membrane; EGFR-positive signal when in the cell membrane and cytoplasm, and VEGF-positive signal when in the cytoplasm. The positive status was further graded according to the proportion of positive cells as described in the Beesley classification method and the staining intensity, with a stained cell count =0 or <10% as negative, 10-25% as (+), 26-50% as (++), and >50% as (+++). The D2-40 interpretation was performed according to the Weidner method (21) in a select region with the highest density of lymphatic vessels at low magnification, where a single endothelial cell or a cluster of endothelial cells stained brownish-yellow represented a positive lymphatic vessel. The numbers of lymphatic vessels in five random fields under the light microscope (×200) were then counted and averaged as the micro-lymphatic vessel density (MLVD). All of the sections were reviewed by senior pathologists in a double-blind fashion.

Statistical analysis

The data were processed in SPSS10.0 software using Chi-square tests to compare intragroup differences. A P value less than 0.05 was considered statistically significant.

Results

Expression of CK19, galectin-3, VEGF and EGFR in the two kinds of thyroid tissue

In tissues of papillary thyroid carcinoma (papillary carcinoma), CK19 expression was mainly found in the cell membrane and cytoplasm, with a positive rate of 100% (*Figure 1A*), whereas in papillary thyroid hyperplasia (papillary hyperplasia), the CK19 positive rate was only 9.1%. The difference between the two groups was significantly different (P<0.05). All specimens of papillary carcinoma with lymph node metastasis showed moderate to intense staining with a positive rate of 100%, and those without lymph node metastasis had similar staining intensity with a same positive rate of 100%, without difference between groups (P>0.05). The positive expression of galectin-3 was mostly in the cytoplasm in papillary carcinoma (*Figure 1B*), with a higher positive rate of 97.4% than that of 16.7% in papillary hyperplasia (P<0.05). Similarly, all specimens of papillary carcinoma with lymph node metastasis showed moderate to highly intense staining with a positive rate of 100%, and those without lymph node metastasis had a positive rate of 96.9%, without significant difference (P>0.05). VEGF, mainly located in the cytoplasm, had mild to intense staining in papillary carcinoma (*Figure 1C*), with a positive rate of 78.9%, higher than that in papillary hyperplasia of 25.0% (P<0.05). The positive rate in the group of papillary carcinoma with lymph node metastasis (83.3%) was higher than that of papillary carcinoma without lymph node metastasis (78.1%, P<0.05). Positive EGFR, mostly in the cell membrane and cytoplasm, showed mild to intense staining in papillary carcinoma (*Figure 1D*), with a positive rate of 73.7%; higher than that of 20.0% (P<0.05). The positive rate in the group of papillary carcinoma with lymph node metastasis (83.3%) was higher than that of papillary carcinoma without lymph node metastasis (71.9%) (P<0.05) (*Table 1*).

D2-40 expressions

Positive expression of D2-40 was located in the membrane and cytoplasm of lymphatic endothelial cells. Since stained micro-lymphatic vessels (MLV) did not primarily exist in papillary thyroid carcinoma, the staining of the stroma and capsule should be taken into account. The micro-lymphatic vessel density (MLVD) in the 38 cases of papillary carcinoma was (13.8±3.7) per field (*Figure 2*), and that in the 12 cases of papillary hyperplasia was (4.3±2.34) per field, with a significant difference between the two groups (P<0.05). The MLVDs of the 6 cases of papillary carcinoma with lymph node metastasis and 32 cases without lymph node metastasis were respectively (14.4±2.13) and (8.6±3.21) per field, with a significant difference between the two groups (P<0.05) (*Table 2*).

Combined test results for D2-40, CK19, galectin-3, VEGF and EGFR expressions

Of the 38 cases with papillary carcinoma, 28 subjects (73.7%) were simultaneously positive for CK 19, galectin-3, VEGF and EGFR, with a MLVD of (13.8±3.7) per field in D2-40 expression. Only one in the 12 cases (8.3%) with thyroid papillary hyperplasia was positive for the above indicators, with a MLVD of (4.3±2.34) per field in D2-40 expression. The difference was significant (P<0.05). Of the six subjects of papillary carcinoma with lymph node metastasis, five were positive for both VEGF and EGFR, accoutning for 83.3% (5/6), with a MLVD of (14.4±2.13) per field in D2-40 expression. Of the 32 subjects of papillary carcinoma without lymph node metastasis, 29 were positive for both VEGF and EGFR, accounting for 71.9% (23/32), with a MLVD of (8.6±3.21) per field. The intragroup differences in both proportions were significant (P<0.05). The positive rates of CK19 and galectin-3 expressions in papillary carcinoma patients with and without lymph node metastasis were 100% (6/6), 100% (6/6), 100% (32/32) and 96.9% (31/32), respectively, without significant difference between the two groups (P>0.05).

Discussion

Lymph node metastasis plays a critical role in the determination of staging, treatment options and prognosis of papillary thyroid carcinoma. Although immunohistochemistry has been widely recognized as an effective adjunct tool for the detection, there has been controversy over the most effective antibody or the need for combined use of antibodies.

The usefulness of CK19 in diagnosing PTC has been studied extensively (11,12), with most studies showing

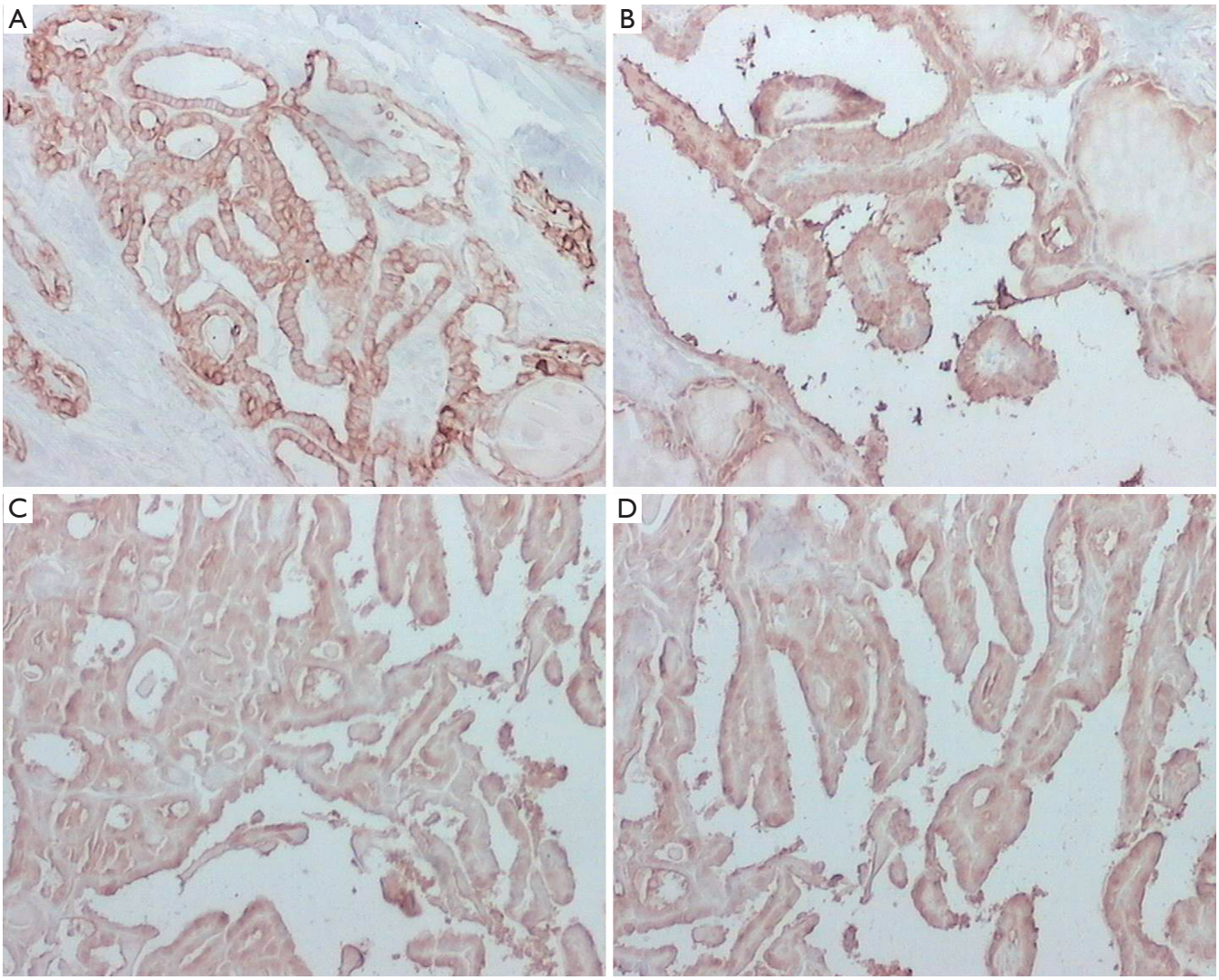

Figure 1 Expressions of CK19, galectin-3, VEGF, and EGFR in papillary thyroid carcinoma (SP ×400). A: CK19; B: galectin-3; C: VEGF; D: EGFR.

Table 1 Expressions of CK19, galectin-3, VEGF, and EGFR in papillary thyroid carcinoma and thyroid papillary hyperplasia

Tissue	n	CK19				Positive rate (%)	Galectin-3				Positive rate (%)	VEGF				Positive rate (%)	EGFR				Positive rate (%)
		(-)	(+)	(++)	(+++)		(-)	(+)	(++)	(+++)		(-)	(+)	(++)	(+++)		(-)	(+)	(++)	(+++)	
Thyroid papillary hyperplasia	12	11	1	0	0	(9.1)*	10	2	0	0	(16.7)*	9	1	2	0	(25.0)*	10	2	0	0	(20.0)*
Papillary thyroid carcinoma	38	0	0	12	26	(100)	1	1	22	14	(97.4)	8	4	18	8	(78.9)	10	3	15	10	(73.7)
With lymph node metastasis	6	0	0	2	4	(100)	0	0	1	5	(100)	1	2	3	0	(83.3)	1	3	2	0	(83.3)
Without lymph node metastasis	32	0	0	10	22	(100)	1	1	21	9	(96.9)	7	2	15	8	(78.1)**	9	0	13	10	(71.9)**

Note: *P<0.05, compared with papillary thyroid carcinoma group; **P<0.05, compared with "with lymph node metastasis" group.

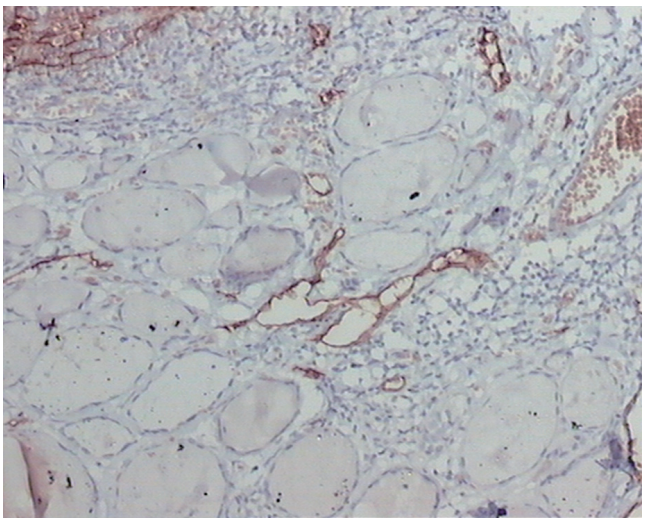

Figure 2 Expression of D2-40 in papillary thyroid carcinoma (SP×100).

Table 2 Relationship between thyroid papillary hyperplasia/ papillary thyroid carcinoma and MLVD

Group	n	MLVD (per field) ($\bar{x}\pm s$)	P
Thyroid papillary hyperplasia	12	4.30±2.34	<0.05
Papillary thyroid carcinoma	38	13.80±3.70	
With lymph node metastasis	6	14.40±2.13	
Without lymph node etastasis	32	8.60±3.21	

strong diffuse expression of CK19 in the majority of PTCs (22,23). In this study, the positive rates of CK19 expression were 100% in papillary carcinoma, 9.1% in papillary hyperplasia (P>0.05), and 100% in papillary carcinoma either with or without lymph node metastasis. Those findings suggested that CK19 was a highly sensitive indicator of thyroid papillary carcinoma, which could be used for the differential diagnosis between papillary carcinoma and papillary hyperplasia, though it added little value to prediction of lymph node metastasis associated with the tumor.

A member of the lectin protein family, galectin-3 is a polypeptide consisting of the amino acid terminal region and the carbohydrate identification zone at the hydroxyl terminal, providing a new option for the detection of angiogenesis (24). Studies have shown that galectin-3 are key to the differential diagnosis of papillary thyroid carcinoma (25). In this study, the positive rates of galectin-3 were 97.4% in papillary thyroid carcinoma and 16.7% in papillary hyperplasia, though as high as 100% and 96% in papillary carcinoma with and without metastasis, respectively. Hence, we believed that galectin-3 could be a valuable antibody for the differential diagnosis of papillary thyroid carcinoma and papillary hyperplasia (26,27), though it was of little significance in predicting lymph node metastasis.

VEGF is a vascular endothelial cell-specific heparin-binding growth factor that can induce angiogenesis in vivo. As a highly conserved homodimeric glycoprotein, it promotes the proliferation of vascular endothelial cells and increases vascular permeability by specifically binding to the three vascular endothelial growth factor receptors (VEGFR-1, -2 and -3). Fellmer and coworkers (28) noted a significantly increased level of VEGF expression in papillary thyroid carcinoma, which was closely linked with the lymph node metastasis yet negatively or weakly present in papillary hyperplasia. In this study, the positive rates of VEGF expression were 78.9% in papillary carcinoma, 25.0% in papillary hyperplasia, and 83.3% and 78.1% in papillary carcinoma with and without lymph node metastasis, respectively, consistent with the findings in the study by Zhang *et al*. (29). We believed that VEGF could induce tyrosine kinase phosphorylation of the lymphatic endothelial cell receptor VEGFR-3 via paracrine or autocrine signaling. The resultant proliferation or expansion of the lymphatic vessels inside or surrounding solid tumors (30) created an open access for invasion by tumor cells and contributed to lymph node metastasis in the region dominated by malignant cells. It could also explain the trend toward lymph node metastasis in papillary thyroid carcinoma. Hung (31) noted a significantly higher level of VEGF expression in papillary thyroid carcinoma with increased potential for lymph node metastasis than in normal tissues and papillary hyperplasia lesions, suggesting that VEGF could be used as an indicator for differentiation between papillary thyroid carcinoma and papillary hyperplasia, as well as the presence of regional lymph node metastasis.

EGFR is the product of the expression of proto-oncogene C-erbB-1, a membrane protein with a molecular weight of 170 kD that consisted of an extracellular functional area for binding to EGF, a short transmembrane domain and an intracellular component that has tyrosine acid activity. EGFR are highly effective in activating tyrosine kinase, and can also exert the activity of this enzyme (32) to cause residue phosphorylation of a specific tyrosine. Through kinase cascade amplification, the signals caused a series of biological effects such as activation of protein kinase and increased synthesis of proteins and DNA, eventually leading

to cell growth and division. As shown in this study, the positive rates of EGFR expression were 73.7% in papillary thyroid carcinoma, 20% in papillary thyroid hyperplasia, and 83.3% and 71.9% in papillary carcinoma with and without lymph node metastasis, respectively, suggesting significantly increased EGFR expression in thyroid papillary carcinoma compared to papillary thyroid hyperplasia. That pattern of EGFR overexpression in tumor tissues was also supported by the study results of Haugen (33). Of all subjects positive for papillary thyroid carcinoma, the expression was also higher in cases with lymph node metastasis than those without, suggesting a close link between EGFR expression and the metastasis.

D2-40 is thought to be a specific marker for lymphatic vessels. Because D2-40 has shown selective immunoreactivity for lymphatic endothelium, its proposedc linical uses include testing for lymphatic invasion by primary tumors (34). As shown in this study, D2-40 specifically located in the lymphatic endothelial cells of the thyroid tissue, making it a more accurate and sensitive indicator applicable for a wider range compared to other lymphatic markers. That was consistent with the findings of Fukunaga (35). Tumor-induced lymphangiogenesis is an important step in lymphatic metastasis. Newborn lymphatic vessels are composed of monolayer cells. The absence of the basal layer, tight junctions between cells, a large number of potential lacunae and other factors are conducive to the entry of tumor cells into the lymphatic vessels. Lymphangiogenesis and the resultant increased lymphatic vessel density contribute to the invasion of tumor cells into regional lymph nodes through lymphatic capillaries, and thus the occurrence of lymphatic metastasis. In this study, the MLVD was significantly higher in papillary carcinoma labeled with D2-40 than in papillary hyperplasia, and also higher in tumors with lymph node metastasis than those without. D2-40 is a more sensitive and specific indicator of tumor lymphatic vessel density and has higher reliability for predicting lymph node metastasis. It is expected to become an indicator for differential diagnosis of papillary thyroid carcinoma and papillary hyperplasia and predictor for lymph node metastasis.

Some studies (22,36) suggested a combination of valuable antibodies to improve the sensitivity and specificity of the differential diagnosis of thyroid cancer. In this study, the expressions of five antibodies (D2-40, CK19, galectin-3, VEGF and EGFR) were significantly higher in papillary carcinoma than in papillary hyperplasia; the expressions of D2-40, VEGF and EGFR were higher in papillary carcinoma with lymph node metastasis than in those without the metastasis, but there was no different in the expressions of CK19 and galectin-3. In conclusion, the combination of D2-40, CK19, galectin-3, VEGF and EGFR is more accurate in the differential diagnosis of papillary carcinoma and papillary hyperplasia and the prediction of lymph node metastasis than a single antibody or double antibody combination. Hence, it is believed that the combination of the five antibodies could be a more reliable tool for the differential diagnosis than single-antibody detection.

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