# Serum Immunoproteomics Combined With Pathological Reassessment of Surgical Specimens Identifies TCP-1ζ Autoantibody As a Potential Biomarker in Thyroid Neoplasia

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**Context:** Current methods of preoperative diagnostics frequently fail to discriminate between benign and malignant thyroid neoplasms. In encapsulated follicular-patterned tumors (EnFPT) this discrimination is challenging even using histopathological analysis. Autoantibody response against tumor-associated antigens (TAA) is a well-documented phenomenon with prominent diagnostic potential; however, autoantigenicity of thyroid tumors remains poorly explored.

**Objectives:** Exploration of TAA repertoire of thyroid tumors and identification of candidate autoantibody biomarkers capable of discrimination between benign and malignant thyroid neoplasms.

**Design, Setting and Patients**

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Abbreviations: 2D two-dimensional; BC Bethesda category; CAP college of American pathologists; CCS colloidal Coomassie stain; CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; CI capsular invasion or confidence interval (clear in context); cPTC papillary thyroid carcinoma, classic variant; DSn diagnostic sensitivity; DSp diagnostic specificity; DTT dithiothreitol; EnFPT encapsulated follicular-patterned tumors; EnFV-PTC papillary thyroid carcinoma, follicular variant, encapsulated; EnWDC-NOS encapsulated well-differentiated carcinomas, not otherwise specified; ESI electrospray ionization; FNAC fine-needle aspiration cytology; FPT follicular-patterned tumors; FTA follicular thyroid adenoma; FTC follicular thyroid carcinoma; FV-PTC papillary thyroid carcinoma, follicular variant; H&E hematoxylin and eosin; HC Hürthle cell features; HT Hashimoto thyroiditis; IEF isoelectric focusing; LC-MS/MS liquid chromatography-tandem mass-spectrometry; NC nitrocellulose; PAGE polyacrylamide gel electrophoresis; PSS Ponceau S stain; PTC papillary thyroid carcinoma; PTC-NC papillary thyroid carcinoma nuclear changes; SDS sodium dodecyl sulphate; SERPA serological proteome analysis; TAA tumor-associated antigen; TRiC tail-less complex polypeptide 1 (TCP-1) ring complex; VI vascular invasion; WB western blot

**Proteins isolated from FTC:** 133 cells were subjected to 2-dimensional Western blotting using pooled serum samples of patients originally diagnosed with either papillary thyroid carcinoma (PTC) or EnFPT represented by apparently benign FTA, as well as healthy individuals. Immunoreactive proteins were identified using LC-MS/MS. Pathological reassessment of EnFPT was performed applying non-conservative criteria for capsular invasion (CI) and significance of focal PTC nuclear changes (PTC-NC). Recombinant TCP-1ζ was used to examine an expanded serum sample set of patients with various thyroid neoplasms (n=89) for TCP-1ζ autoantibodies. All patients were included in tertiary referral centers.

**Results:** A protein demonstrating a distinct pattern of EnFPT-specific seroreactivity was identified as TCP-1ζ protein. Subsequent search for clinico-pathological correlates of TCP-1ζ seroreactivity revealed non-classical CI or focal PTC-NC in all TCP-1ζ antibody-positive cases. Further studies in expanded sample set confirmed the specificity of TCP-1ζ autoantibodies to malignant EnFPT.

**Conclusions:** We identified TCP-1ζ autoantibodies as a potential biomarker for pre-surgical discrimination between benign and malignant encapsulated follicular-patterned thyroid tumors. Our results suggest the use of non-conservative morphological criteria for diagnosis of malignant EnFPT in biomarker identification studies and provide a peculiar example of uncovering the diagnostic potential of a candidate biomarker using incorporation of pathological reassessment in the pipeline of immunoproteomic research.

Most thyroid neoplasms arise from follicular epithelia and may be tentatively divided into papillary thyroid carcinomas (PTC), follicular thyroid carcinomas (FTC) and benign follicular thyroid adenomas (FTA). Numerous histological subtypes of these tumors including hybrid phenotypes (ie, follicular variant of papillary thyroid carcinoma, FV-PTC) as well as oncocytic (oxyphilic) subtypes harboring additional disruptive mutations in mitochondrial DNA (1) have been identified (2). FTA, FTC, encapsulated FV-PTC (EnFV-PTC) and encapsulated well-differentiated carcinomas, not otherwise specified (NOS) (EnWDC-NOS) (including their variants), may be collectively referred to as «encapsulated follicular-patterned tumors» (EnFPT) based on common architectural, clinical and molecular features distinct from those of classic PTC (3, 4).

Fine-needle aspiration cytology (FNAC) is able to correctly classify most PTC cases based on characteristic PTC nuclear changes (PTC-NC) (5, 6), and accurate exclusion of malignancy may also be achieved in most non-neoplastic lesions, such as adenomatous nodules or lymphocytic (Hashimoto) thyroiditis (HT) (7, 8). However, FNAC is not able to discriminate between FTA and FTC both lacking PTC-NC but differing in their invasive characteristics and propensity to metastasize (9, 10). A correct preoperative diagnosis in a large number of FV-PTC cases also remains a challenging task, since PTC-NC in these neoplasms are frequently incomplete and focal (11). In the absence of widely invasive growth, differential diagnosis between benign and malignant EnFPT may become difficult even in routine histopathology and is a subject of dramatic observer variations concerning thresholds to be used for recognizing the capsular invasion (CI) and the significance of focal PTC-NC in otherwise benign tumors (3, 12–14). Thus, there is a need for novel biomarkers of malignancy in EnFPT. Such biomarkers should allow overcoming of unnecessary «diagnostic surgery» procedures while maintaining the excellent long-term results of treatment.

Autoantibodies against tumor-associated antigens (TAA) are spontaneously produced in cancer patients and much effort has been made to identify their antigenic targets. Numerous TAA and autoantibody signatures were identified in association with many malignant tumors (reviewed in Refs (15–17).), however, endocrine tumors and thyroid neoplasms in particular remain poorly explored in this context (18). In the present study, we used ***ser***ological ***p***roteome ***a***nalysis (SERPA) (19) to identify TCP-1ζ protein as an antigen eliciting frequent autoantibody responses in EnFPT. Although initially TCP-1ζ autoantibodies were found in patients diagnosed with apparently benign FTA, subsequent pathological reassessment demonstrated the presence of minor foci of CI or PTC-NC in all TCP-1ζ antibody-positive cases, thus implicating TCP-1ζ as a cancer-associated antigen. Further studies using expanded sample set revealed that TCP-1ζ serological reactivity is virtually restricted to EnFPT and demonstrates a 100% predictive value for malignancy in these neoplasms. Taken together, we identified TCP-1ζ autoantibodies as a potential auxiliary biomarker for differential diagnosis between benign and malignant thyroid EnFPT. Our study also suggests that utilization of non-conservative morphological threshold for diagnosis of malignancy may be important for successful identification of biomarker candidates, especially in tumors lacking overtly invasive behavior and clear-cut nuclear atypia.

## Materials and Methods

### Cell line and total protein extract preparation

Follicular thyroid carcinoma cell line FTC-133 was purchased from Health Protection Agency Culture Collection (Salisbury, UK) and cultured in combined media containing DMEM: Ham's F12 1:1 supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 100 I.U/ml penicillin/100 $\mu$g/ml streptomycin. Subconfluent cells were scraped off, washed 3 times with ice-cold PBS followed by solution containing 10 mM Tris-HCl and 250 mM sucrose pH 7.0, lysed with modified Rabilloud IEF/lysis buffer containing 7M urea, 2M thiourea, 0.25% (v/v) carrier ampholytes pH 3–10 (Bio-Rad Pharmalytes), 2% (w/v) CHAPS, 43 mM DTT and protease inhibitor cocktail (Roche) and sonicated on ice. Protein extract was centrifuged, supernatant was collected, aliquoted and frozen at –70°C.

### Patients and serum samples

Patients' blood samples were collected before or at the time of thyroid surgery between 2007 and 2009 in Surgical Department of Endocrinology Research Center (Moscow, Russia). The FNA biopsy preceded the blood collection by a median of 36 days (range 5–259 days) for benign EnFPT; 49 days, (range 6–165 days) for malignant EnFPT; and 53 days (range 5–140 days) for infiltrative PTC (Mann-Whitney U-test p-values $> 0.2$ in all pairwise comparisons). FNAC records were retrospectively retrieved from the institutional database and rendered compliant with the Bethesda System for Reporting Thyroid Cytopathology (9). FNAC categories follicular neoplasm (Bethesda IV), suspicious for papillary thyroid carcinoma (Bethesda V) and papillary thyroid carcinoma (Bethesda VI) were eligible.

The total protein content of sera was determined as an integral part of blood biochemistry panel at the routine preoperative work-up, returning the values within the reference range of 60–87 mg/ml for all patients included in the study. The study was approved by Ethical Review Board of Endocrinology Research Center.

Additional serum samples (10 cPTC and 6 malignant EnFPT cases, comprising 2 EnFV-PTC and 4 encapsulated well-differentiated carcinomas, NOS (EnWDC-NOS)) were obtained from the Medical Radiology Research Center (Obninsk, Russia). The cases were selected from the institutional database in the way that the age at surgery and tumor size were within the ranges of cPTC and malignant EnFPT groups comprising the main sample set.

Control sera were obtained from healthy females (median age 34, range 24–62) without palpable thyroid nodules during the routine annual medical examination in the outpatient clinic of the Moscow State University (Moscow, Russia).

All individuals mentioned hereinbefore provided informed consent. Sera were isolated, aliquoted and frozen at –70°C. For serological proteome analysis, sera were pooled (5 samples in each pool) according to the initial histological diagnosis to obtain 2 classical PTC (cPTC), 2 FTA and 2 healthy (HD) pooled samples.

### Histopathological analysis

Archival H&E stained slides were retrieved from the archive of Department of Pathomorphology of Endocrinology Research Center; if slides were not available, FFPE tissue blocks were retrieved, 5 $\mu$m sections were prepared and H&E-stained using standard method. Slides were independently reviewed by two surgical pathologists specifically trained in thyroid pathology (A.Y.A. and N.Y.D.) using College of American Pathologists (CAP) protocol criteria for CI and VI (20) and FV-PTC classification proposed by LiVolsi and Baloch (11) as detailed in Supplemental Methods. The consensus pathology was then established. Cases were excluded if the principal consensus (benign vs malignant) was not reached and if PTC-NC in noninvasive tumor could not be evaluated (eg, only frozen section was available for review).

### Serological proteome analysis

FTC-133 protein lysate was subjected to isoelectric focusing (Protean IEF cell, Bio-Rad) using 7-cm immobilized pH gradient strips with pH gradient 3–10/3–10 NL (Bio-Rad). Following focusing up to 33 kVh strips were equilibrated in 6M urea, 0.375M Tris-HCl pH 8.8, 2% (w/v) SDS, 20% (w/w) glycerol and 1% (w/v) DTT for 20 minutes and subjected to SDS-PAGE in the second dimension using 10% polyacrylamide gels. Gels were either stained with colloidal Coomassie Brilliant Blue G-250 stain (CCS) or electroblotted onto nitrocellulose (NC) membranes (Hybond-C Extra, Amersham Biosciences). Following transfer and Ponceau S staining (PSS), membranes were blocked (5% skim milk in TBS-T (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.1% (v/v) Tween-20)) and incubated overnight at +4°C on a rotary shaker with pooled sera diluted 1:300 in blocking solution. Membranes were washed 4 times in TBS-T, followed by 1 hour incubation with horseradish peroxidase-labeled secondary antibodies (Pierce, goat anti human IgG, Fc$\gamma$ fragment-specific) diluted 1:10000 in blocking solution. After final wash (4 $\times$ 15 minutes in TBS-T) chemiluminescent signal was generated using SuperSignal West Pico Chemiluminescent Substrate (Pierce), and blots were placed against the X-ray film (Kodak MXB) for 30 seconds. Scans of the films were imported into Adobe Photoshop software and superimposed with those of respective PSS membranes which were used for visual WB/PSS spot matching. The recurrent FTA class-specific spot 9FS was localized on the scan of colloidal Coomassie-stained gel using further superimposition with the WB/PSS overlay, excised from the CCS gel and subjected to LC-ESI-MS/MS analysis as described in Supplemental Methods.

### 1-dimensional Western blot analysis

Recombinant TCP-1$\zeta$ preparation (see Supplemental Methods for details) was separated in 12.5% polyacrylamide gels, electroblotted onto NC membranes and blocked as described above. Resulting membranes were cut into strips (1 lane per strip) and probed with individual patients' sera diluted as specified below in blocking solution and premixed with a cleared lysate of BL-21(DE3) *E.coli* strain (up to 2 mg/ml) to ensure the absence of any possible serological reactivity against minor bacterial protein contaminants. Subsequent technical workflow was identical to that described in previous section.

For initial testing of sera comprised original pooled samples, 2 $\mu$g of antigen per lane and serum dilution 1/500 were used. Films were exposed to blots for 5 to 10 seconds and samples generating any visible band corresponding to full-length TCP-1$\zeta$ were considered positive.

For determination of TCP-1$\zeta$ antibody frequency and quantitative analysis, adjusted conditions of 4 $\mu$g of antigen per lane and 1/1500 serum dilution were used (case FN30 was further

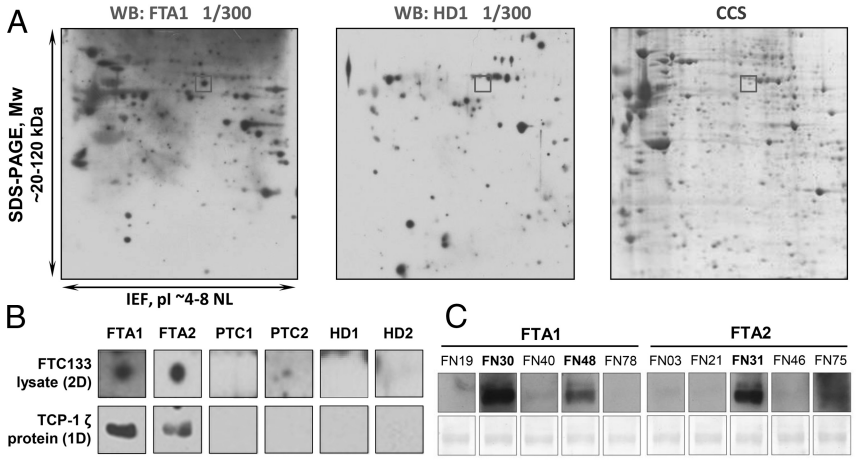


**Figure 1.** ***Identification of 9FS spot with FTA-specific reactivity.*** (A) Representative 2D images corresponding to enhanced chemiluminescence WB replicas of membranes hybridized with FTA1 (follicular thyroid adenoma) and HD1 (healthy individuals) pooled samples diluted 1/300, as well as colloidal Coomassie-stained gel (CCS); spot 9FS is marked with rectangular frame; (B) Close-up sections of WB images corresponding to the 9FS spot area. 2D blots of FTC-133 proteins and 1D blots of recombinant human (rh) TCP-1ζ were probed with the indicated pooled serum samples (upper and lower panels, respectively, 1/300 pooled sample dilution). FTA – follicular thyroid adenoma, PTC – papillary thyroid carcinoma, classic variant, HD – healthy donors; (C) Close-up sections corresponding to 1D blots of recombinant TCP-1ζ protein probed with individual sera comprising FTA1 and FTA2 pooled samples (1/500 serum dilution); reactive sera' IDs are in bold; corresponding sections of Ponceau S-stained membranes are also shown (loading controls).

analyzed at 1/3000 serum dilution since even 1/1500 dilution and minimal film exposure resulted in a saturated signal). At least 2 positive and 2 negative controls (selected from the sample set comprising the original pooled samples) were included in each experiment. Films exposed to blots for 10, 15 and 20 seconds were scanned at 300 pixels/inch using Epson Perfection 4870 PHOTO scanner without image enhancement and saved as non-compressed grayscale TIFF files. All images were analyzed using ImageJ software (http://imagej.nih.gov/ij/) as described in Supplemental Methods. Each serum was tested in at least 2 independent experiments (Supplemental Figure 2).

### Statistical analysis

The Fisher's exact test (http://www.quantitativeskills.com/sisa/statistics/fisher.htm) was implemented for testing of significance of most serological reactivity's associations, with the exception of age and tumor size variables that were assessed using Mann-Whitney U-test (*http://vassarstats.net/utest.html*). P-values $< 0.05$ were considered significant. P-values reported are two-tailed and uncorrected unless specifically indicated otherwise. Confidence intervals for DSn and DSp values were calculated using web-based calculator http://vassarstats.net/clin1.html

## Results

### Immunoproteomic identification of TCP-1ζ protein as an antigen recognized by serum autoantibodies in thyroid tumor patients

We applied modified SERPA (19) analysis using FTC-133 follicular carcinoma cell line as an antigen source. FTC-133 proteins were separated using two-dimensional gel electrophoresis, blotted onto nitrocellulose membranes and probed with pooled serum samples as described in Materials and Methods. One particular protein (spot 9FS) demonstrating FTA-restricted pattern of serological reactivity (Figure 1A-B) was subsequently identified using tandem mass-spectrometry as a ζ subunit of a tail-less complex polypeptide 1 (TCP-1) ring complex (TRiC) encoded by human *CCT6A* gene (Supplemental Figure 1). Reactivity of recombinant $His_6$-tagged TCP-1ζ protein in Western-blot analysis supported the initially observed pattern (Figure 1B), further confirming the identity of the target antigen as human TCP-1ζ. Case-by-case analysis of TCP-1ζ autoantibodies in individual sera comprising original pooled samples revealed 3 out of 10 strongly reactive sera in FTA group (patients FN30, FN31 and FN48, Figure 1C). Healthy individuals (n = 10) and cPTC sera (n = 10) comprising the original pooled samples showed no reactivity against recombinant TCP-1ζ (data not shown).

### Pathological reassessment of EnFPT cases implicates TCP-1ζ autoantibodies as a potential biomarker of malignancy in thyroid EnFPT

We noticed that all 3 tumors in TCP-1ζ autoantibody positive patients measured ≥ 25 mm, compared with only 1/7 such tumors among TCP-1ζ autoantibody negative patients ($P = .033$, starred cases in Table 1 and case FN75 (8 mm) excluded from further analysis, see below). Tumor size is a strong predictor of malignancy in many human neoplasms, and this is also the case for thyroid tumors (21–24). Thus we decided to reinvestigate the archival histological specimens of these tumors in an attempt to identify any features of malignancy that might have been rendered as incomplete/questionable findings during the initial pathological examination.

Surprisingly, we found that all 3 tumors of TCP-1ζ-seropositive patients initially classified as «benign» contained features of low-grade malignancy (Figure 2). In all 3 cases, tumor nodules were completely encapsulated and were initially classified as «follicular adenomas». In case FN30, the tumor demonstrated incomplete PTC-NC throughout the lesion with multiple foci of full-blown PTC

**Table 1.** Basic clinico-pathological characteristics of 34 EnFPT patients

| ID | Age/Gender | FNAC BC | Tumor size | HC | PTC-NC | CI | VI | HT | TCP-1ζ Abs |
|---|---|---|---|---|---|---|---|---|---|
| **Follicular Thyroid Adenomas (FTA)** | | | | | | | | | |
| **FN01** | **69/M** | **V** | **22** | **-** | **-** | **-** | **-** | **-** | **-** |
| **FN03*** | **48/F** | **IV** | **60** | **-** | **-** | **-** | **-** | **-** | **-** |
| **FN04** | **64/F** | **V** | **32** | **+** | **-** | **-** | **-** | **-** | **-** |
| FN06 | 66/F | IV | 30 | - | - | - | - | + | - |
| FN11 | 31/F | IV | 12 | - | - | - | - | - | - |
| FN19* | 52/F | IV | 15 | - | - | - | - | + | - |
| FN21* | 67/F | V | 21 | - | - | - | - | - | - |
| FN28 | 52/F | V | 13 | + | - | - | - | - | - |
| FN40* | 33/F | IV | 20 | - | - | - | - | - | - |
| FN46* | 22/M | IV | 15 | - | - | - | - | + | - |
| FN47 | 66/F | IV | 10 | - | - | - | - | + | - |
| FN50 | 29/F | IV | 36 | + | - | - | - | + | - |
| FN51 | 50/F | IV | 35 | + | - | - | - | - | - |
| FN56 | 39/M | IV | 22 | - | - | - | - | - | - |
| FN78* | 54/F | IV | 21 | - | - | - | - | + | - |
| **Encapsulated follicular variant of papillary thyroid carcinomas (EnFV-PTC)** | | | | | | | | | |
| **FN02** | 48/F | IV | 44 | + | 2+ | + | - | - | - |
| FN05 | 63/F | IV | 25 | + | 2+ | - | - | - | + |
| **FN30*** | 43/F | IV | 25 | - | 2+ | - | +† | - | + |
| **FN37** | 46/F | IV | 23 | + | 2+ | + | - | + | - |
| **FN44** | 23/F | IV | 36 | - | 2+ | - | - | - | - |
| **FN48*** | 51/F | IV | 28 | - | + | - | - | + | + |
| FN83 | 48/F | VI | 24 | - | 2+ | 2+ | + | + | - |
| FN96 | 33/F | NA | 50 | - | 2 + + | - | - | - | - |
| FN97 | 36/F | NA | 50 | - | 2 + + | + | - | - | - |
| **Encapsulated well-differentiated carcinomas, not otherwise specified (EnWDC-NOS)** | | | | | | | | | |
| FN14 | 37/M | V | 24 | - | NE | 2+ | + | - | - |
| **FN82** | 58/F | IV | 20 | + | NE | 2+ | - | - | + |
| FN98 | 25/F | NA | 25 | - | Eq | + | - | - | + |
| FN99 | 30/F | NA | 26 | - | Eq | 2+ | - | + | - |
| FN100 | 31/F | NA | 34 | - | Eq | 2+ | - | - | - |
| FN101 | 26/F | NA | 18 | - | Eq | + | - | - | + |
| **Follicular Thyroid Carcinomas (FTC)** | | | | | | | | | |
| **FN31*** | 41/F | IV | 48 | - | - | 2+ | - | - | + |
| FN55 | 44/F | IV | 26 | - | - | 2+ | - | - | - |
| FN59 | 42/F | IV | 70 | + | - | 2+ | + | - | - |
| FN43 | 60/M | IV | 37 | - | - | 2+ | + | - | + |

BC – Bethesda Category of FNA cytology (IV – follicular neoplasm or suspicious for follicular neoplasm, V – suspicious for malignancy, VI – malignant). CI – capsular invasion, VI – vascular invasion, PTC-NC – papillary thyroid carcinoma nuclear changes, HC – Hürthle cell features, HT – Hashimoto thyroiditis as revealed by histopathological analysis of non-tumorous thyroid tissues. CI and PTC-NC were scored according to the following: CI: +: incomplete invasion into but not through the fibrous capsule; 2+: at least one focus of through-and-through capsular penetration. PTC-NC: +: a single microfocus of full-blown PTC-NC in otherwise benign FTA; 2+: multiple foci of full-blown PTC-NC interspersed with regions demonstrating either totally bland nuclei or incomplete PTC-NC; 2++: diffusely distributed full-blown PTC-NC; Eq – diffusely distributed equivocal PTC-NC; NE – not evaluable (see Supplemental Methods for details). *Sera used in initial SERPA analysis. †Discovered by deeper sectioning of archival tissue block. Cases whose diagnoses were upgraded upon pathological re-review are marked in bold.

nuclei (Figure 2A); a single focus of vascular invasion was also noted upon deeper sectioning of archival tissue block; the revised diagnosis was EnFV-PTC (type 3 according to LiVolsi and Baloch (11)). In case FN31, the tumor demonstrated a satellite nodule of the identical morphology (Figure 2B) located just outside the main tumor (complete invasion according to CAP Protocol criteria (20)); the revised diagnosis was «minimally invasive FTC». In case FN48, the encapsulated solid/microfollicular adenoma (Figure 2D) demonstrated a subcapsular microfocus with markedly different architecture and cells showing full-blown PTC-NC, suggesting the development of FV-PTC on FTA background (Figure 2C); the revised diagnosis was EnFV-PTC (type 6 according to LiVolsi and Baloch (11)). Only frozen section of noninvasive oxyphilic tumor was available for review in FN75 case; since the PTC-NC were not evaluable under these circumstances, this case was excluded from further analysis. In contrast to TCP-1ζ-

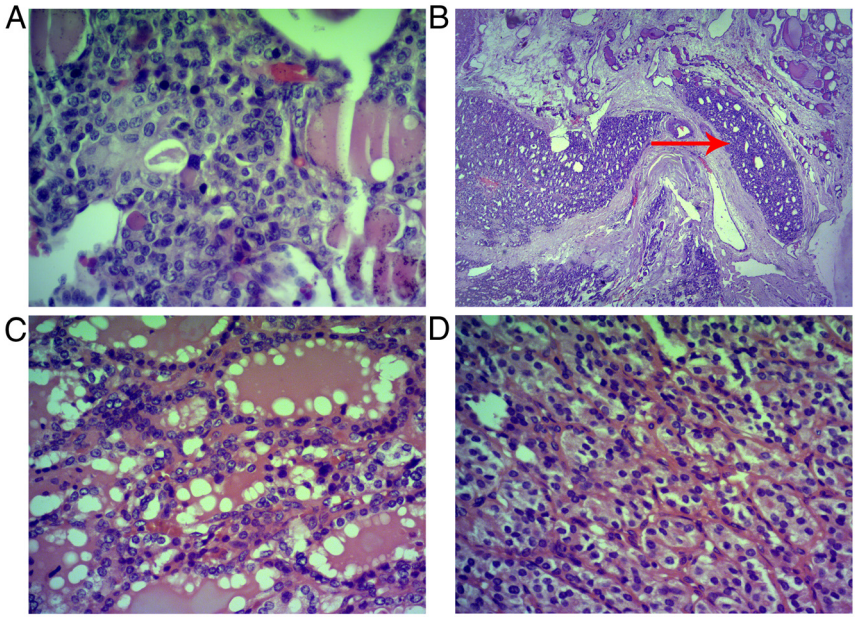


**Figure 2.** ***Histopathological analysis of 3 TCP-1ζ-seropositive cases.*** (A) Case FN30, high-power view of a tumor demonstrating follicular architecture; cells demonstrate typical features of PTC-NC (elongation, clearing, grooving), H&E 400X; (B) Case FN31, low-power view of invasive margin with a satellite nodule morphologically identical to the main tumor located just outside the capsule outwith capsular vessel (red arrow), H&E 40X; (C) Case FN48, high-power view of a subcapsular FV-PTC focus in FTA, the follicles filled with dense scalloped colloid are lined with cells demonstrating abundant nuclear clearing, crowding and occasional grooving, H&E 400X; and (D) Case FN48, representative field of the gross of the tumor with normochromatic nuclei lacking any signs of PTC-NC, H&E 400X.

seropositive patients, none of the remaining 6 tumors in TCP-1ζ autoantibody-negative FTA patients ($P = .012$) demonstrated any signs of capsular or vascular invasion and/or PTC-NC aside from minor capsular irregularities and occasional nuclear clearing in single cells scattered throughout the lesion. Thus, TCP-1ζ emerged as a candidate cancer-associated antigen specific for malignant EnFPT.

### Evaluation of TCP-1ζ serological reactivity in various thyroid neoplasms confirms the specificity of TCP-1ζ autoantibodies to malignant EnFPT

We next proceeded to evaluation of TCP-1ζ antibody frequency using Western blot analysis across the expanded sample set comprising a total of 89 thyroid tumor patients. The sample set comprised 34 EnFPT cases (Table 1), 51 patients diagnosed with various PTC variants distinct from EnFV-PTC and 4 non-FTA benign lesions (Figure 3A and Supplemental Table 2). Prior to the analysis, all tumors in EnFPT subset were subjected to rigorous pathological re-examination as described in Materials and Methods; cases were included only if the final consensus pathology could be established. Besides the 3 cases described earlier, 4 additional tumors were reclassified from FTA to EnFV-PTC (3 cases) and EnWDC-NOS (1 case); among 12 tumors classified as «malignant» on the initial pathological evaluation, none was reclassified, thus resulting in 19 malignant and 15 benign EnFPT cases in the sample set (Table 1).

As expected from our preliminary data, FTA and PTC patients diagnosed with non-EnFV-PTC variants demonstrated very low frequencies of TCP-1ζ autoantibodies; in contrast, patients with malignant EnFPT demonstrated significantly higher frequencies of TCP-1ζ serological reactivity, estimated to be as high as 33%–50% (Figure 3, $P < .05$ for each of EnFV-PTC, EnWDC-NOS, FTC and combined EnFPT groups as compared to FTA group). In the post hoc analysis performed in malignant EnFPT group, none of clinico-pathological factors (the presence of capsular or vascular invasion, age at diagnosis, gender, tumor size, oxyphilia of tumor cells and background lymphocytic thyroiditis) was significantly associated with the presence of TCP-1ζ serum autoantibodies in the malignant EnFPT group (Supplemental Table 1).

We then analyzed the diagnostic performance of TCP-1ζ autoantibodies in presurgical setting. All six TCP-1ζ autoantibody positive patients (for whom the information on initial FNAC conclusion was available) were diagnosed with «follicular neoplasm» FNAC (Bethesda category IV, Table 1). Thus, we focused particularly on this subgroup of patients. All patients with other FNAC categories listed in Table 1 (n = 12) were excluded from the analysis. Among non-EnFPT patients in our 89-sample set, eight had BC IV nodules, 4 being diagnosed with malignant and 4 – with non-FTA benign lesions (Supplemental Table 2); these patients were included in the analysis. The diagnostic specificity (DSp) and sensitivity (DSn) of TCP-1ζ autoantibody in detection of malignancy in BC IV nodules were estimated as 100% (15/15, 95% CI 75%–100%) and 40% (6/15, 95% CI 17%–67%), respectively ($P = .017$).

## Discussion

During last two decades, autoantibodies emerged as a promising tool for diagnostic purposes (16, 17), and the first commercial autoantibody-based test *Early*CDT®-

Lung (http://www.earlycdt-lung.co.uk/) for the early diagnostics of lung cancer has recently entered the market. However, autoantigenic repertoires of many human neoplasms, eg, endocrine tumors, have not been studied in sufficient detail. Only two top-down studies identified several antigens eliciting autoantibody responses in anaplastic (25) and papillary (26) thyroid carcinomas, and few additional antigens were implicated in thyroid carcinomas in bottom-up setting (15, 27–30). Importantly, none of these studies focused on encapsulated follicular-patterned lesions. Here, using pathological reassessment of surgical specimens, we show that TCP-1ζ protein initially identified using immunoproteomics as FTA-associated TAA in fact represents the target of frequent immune response in malignant EnFPT but not in other thyroid neoplasms (Figure 3).

TCP-1ζ is an ubiquitously expressed subunit of TCP-1 ring complex (TRiC) assisting folding of cytosolic proteins. Several reports implicated TCP-1ζ in carcinogenesis (31, 32); its overexpression was demonstrated in human cancers (31–34), whereas Rhodes et al (35) identified *CCT6A* as a part of 69-gene signature commonly upregulated in undifferentiated carcinomas. Interestingly, human *CCT6A* gene is localized to chromosomal region 7p11.2 in the close proximity to *EGFR*. The copy number gain of chromosome 7 genomic material with minimal recurrent

A

| Histopathology | TCP-1ζ autoantibody frequency | |
|---|---|---|
| | Individual groups | Combined subsets |
| FTA | 0/15 | Total benign nodules: |
| Non-FTA benign nodules* | 0/4 | 0/19 (0%) |
| EnFV-PTC | **3/9 (33%)** | Total malignant EnFPT: **8/19 (42%)** |
| EnWDC-NOS | **3/6 (50%)** | |
| FTC | **2/4 (50%)** | |
| PTC, classic variant | 1/38 (3%) | Total non-encapsulated PTC: |
| PTC, other variants† | 0/13 | 1/51 (2%) |

B

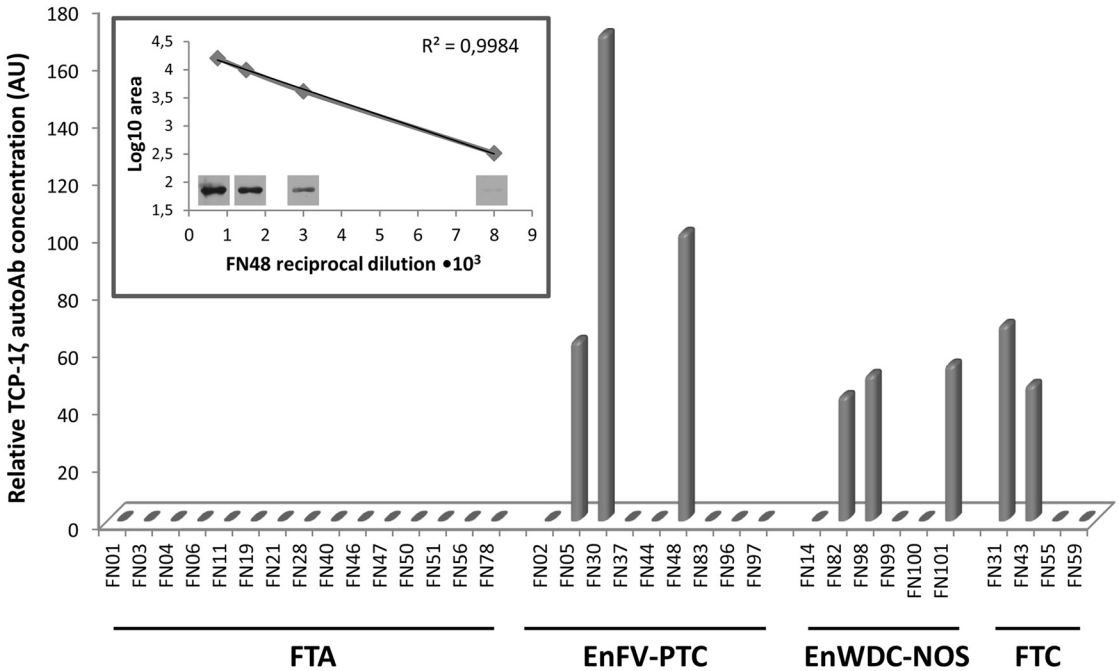


**Figure 3.** ***Western-blot analysis of TCP-1ζ autoantibodies in thyroid nodular lesions.*** (A) Summary of TCP-1ζ serological reactivity in various thyroid nodular lesions. Significant differences ($P < .05$) as compared to the FTA group using two-tailed Fisher exact test are marked in bold. *Nodular hyperplasia (n = 2), Hashimoto thyroiditis (n = 1) and hyalinizing trabecular tumor (n = 1). †Nonencapsulated PTC variants: nonencapsulated follicular (n = 6), solid/follicular (n = 3), Warthin-like (n = 2), diffuse sclerosing (n = 1) and tall cell (n = 1). All sera were tested at 1/1500 dilution. B, Relative quantification of TCP-1ζ autoantibodies in thyroid EnFPT. The calibration curve was established using dilutions of FN48 serum as shown on the side bar and described in Supplemental Methods; the calculated FN48 dilution corresponding to each peak area was then converted to relative concentration expressed as AU=(FN48 dilution corresponding to a peak area)(actual reciprocal dilution)100. All sera were tested at 1/1500 dilution; the AU value for FN30 sera was calculated based on the signal corresponding to 1/3000 dilution (see Materials and Methods for details). Data corresponding to one representative experiment is shown (see also Supplemental Figure 2).

region of gain at 7p12-p11 is a cytogenetic hallmark of EnFPT (36) but not of cPTC (37), suggesting a possible mechanistic link between *CCT6A* gene amplification and autoantibody response against its protein product TCP-1ζ. This cytogenetic distinction also provides a possible explanation for the absence of TCP-1ζ autoantibodies in infiltrative PTC variants. Other oncogenic events specific for EnFPT such as RAS point mutations or PAX8/PPARγ translocations may also indirectly contribute to TCP-1ζ autoantibody response, probably via modulation of TCP-1ζ expression levels.

In the subset of patients with indeterminate FNAC (Bethesda category IV), the TCP-1ζ autoantibodies demonstrated diagnostic performance in detection of malignancy typical for this class of biomarkers being 100% predictive for malignancy (DSp 100%, 95% CI 75%–100%) while showing low-to-moderate DSn (40%, 95% CI 17%–67%). Combinations of several autoantibody biomarkers may significantly improve DSn without sacrificing DSp (38–40), and further studies are ongoing to identify additional autoantigens to be used in combination with TCP-1ζ. The lack of association of TCP-1ζ serological reactivity with Hashimoto thyroiditis (Table 1, Supplemental Table 1 and data not shown) suggests that diagnostic performance of TCP-1ζ autoantibodies is unlikely to be inflated by TCP-1ζ serological reactivity associated with concurrent autoimmune disease, the phenomenon typical for many other TAA (41). All malignant EnFPT in the sample set were non- (n = 4) or minimally (n = 15) invasive (Table 1), indicating that TCP-1ζ autoantibody response occurs at the early stages of «benign-to-malignant» transition. Taken together, our data suggest a prominent diagnostic potential of TCP-1ζ autoantibodies in the most diagnostically challenging group of thyroid neoplasms. To our knowledge, this is the first report of a thyroid cancer-associated TAA that demonstrates differential serological reactivity between benign and malignant thyroid EnFPT, as well as the first implication of TCP-1ζ protein as human TAA.

EnFPT are subject to dramatic observer variations in their classification with respect to diagnostic thresholds used for capsular invasion (CI) and significance of focal PTC-NC, and thus in differential diagnosis between benign (FTA) and malignant (FTC and EnFV-PTC) EnFPT (12–14). Recently, changing of these diagnostic thresholds in a retrospective cohort resulted in the upgrade of the initially benign diagnosis in 35 out 118 (30%) thyroid tumors, raising important ethical as well as scientific issues, eg, the validity of archival pathology records in retrospective studies of thyroid neoplasia (12). In our study, re-examination of archival specimens allowed us to reveal a cancer-associated profile of TCP-1ζ serological reactivity initially obscured due to highly conservative approach for diagnosis of malignancy, rendering focal PTC-NC and single focus of nonclassical CI insignificant. The conservative approach may be convenient in routine pathology practice since post-treatment course of EnFPT lacking vascular invasion seem to be typical for benign rather than malignant tumors with extremely low risk of local and systemic progression (42–45), although exceptions have been described (46–48). However, this approach, oriented primarily on prognosis-guided patient management, almost completely ignores the real biology of these tumors. Particularly, such non/minimally invasive carcinomas (or, according to other designations – «borderline» (44, 45) and «uncertain malignant potential» (49) tumors) are expected to share a large part of their «omic» (genomic, transcriptomic, proteomic, immunomic etc.,) signatures with those of fully invasive malignancies. The failure to identify the former in the sample sets used for biomarker studies may result in considerable data loss and false rejection of promising biomarker candidates. Although natural history of non/minimally invasive malignant EnFPT is virtually unknown, from the biological standpoint they are more likely to progress to fully invasive malignancies, if left untreated, than the *bona fide* benign tumors. Thus, it is also apparently plausible to discriminate between those two entities during initial diagnostic workup, and our study provides a proof-of-principle for the use of autoantibody biomarkers, particularly TCP-1ζ autoantibody, for these purposes.

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## References


1. **Maximo V, Lima J, Prazeres H, Soares P, Sobrinho-Simoes M.** The biology and the genetics of Hurthle cell tumors of the thyroid. *Endocr Relat Cancer*. 2012;19:R131–R147.
2. **DeLellis RA, Williams ED. Tumors of thyroid and parathyroid. In: DeLellis RA, Lloyd RV, Heitz PU, Eng C, eds.** *Pathology and Genetics of Tumors of Endocrine Organs*. Lyon, France: IARC Press; 2004:49–133. *World Health Organization Classification of Tumours*, vol 8.
3. **Baloch ZW, Livolsi VA.** Follicular-patterned lesions of the thyroid: the bane of the pathologist. *Am J Clin Pathol*. 2002;117:143–150.
4. **Livolsi VA, Baloch ZW.** Follicular-patterned tumors of the thyroid: the battle of benign vs. malignant vs. so-called uncertain. *Endocr Pathol*. 2011;22:184–189.
5. **Baloch ZW, Livolsi VA.** Pathologic diagnosis of papillary thyroid carcinoma: today and tomorrow. *Expert Rev Mol Diagn*. 2005;5:573–584.
6. **Das DK.** Intranuclear cytoplasmic inclusions in fine-needle aspiration smears of papillary thyroid carcinoma: a study of its morphological forms, association with nuclear grooves, and mode of formation. *Diagn Cytopathol*. 2005;32:264–268.
7. **Gharib H, Goellner JR, Johnson DA.** Fine-needle aspiration cytology of the thyroid. A 12-year experience with 11,000 biopsies. *Clin Lab Med*. 1993;13:699–709.
8. **Yassa L, Cibas ES, Benson CB, Frates MC, Doubilet PM, Gawande AA, Moore FD, Jr., Kim BW, Nose V, Marqusee E, Larsen PR, Alexander EK.** Long-term assessment of a multidisciplinary approach to thyroid nodule diagnostic evaluation. *Cancer*. 2007;111:508–516.
9. **Cibas ES, Ali SZ.** The Bethesda System For Reporting Thyroid Cytopathology. *Am J Clin Pathol*. 2009;132:658–665.
10. **Cap J, Ryska A, Rehorkova P, Hovorkova E, Kerekes Z, Pohnetalova D.** Sensitivity and specificity of the fine needle aspiration biopsy of the thyroid: clinical point of view. *Clin Endocrinol (Oxf)*. 1999;51:509–515.
11. **LiVolsi VA, Baloch ZW.** The Many Faces of Follicular Variant of Papillary Thyroid Carcinoma. *Pathology Case Reviews* 2009;14.
12. **Widder S, Guggisberg K, Khalil M, Pasieka JL.** A pathologic review of follicular thyroid neoplasms: The impact of changing the threshold for the diagnosis of the follicular variant of papillary thyroid carcinoma. *Surgery*. 2008;144:80–85.
13. **Hirokawa M, Carney JA, Goellner JR, DeLellis RA, Heffess CS, Katoh R, Tsujimoto M, Kakudo K.** Observer Variation of Encapsulated Follicular Lesions of the Thyroid Gland. *The American Journal of Surgical Pathology* 2002;26.
14. **Elsheikh TM, Asa SL, Chan JK, DeLellis RA, Heffess CS, Livolsi VA, Wenig BM.** Interobserver and intraobserver variation among experts in the diagnosis of thyroid follicular lesions with borderline nuclear features of papillary carcinoma. *Am J Clin Pathol*. 2008;130:736–744.
15. **Tan EM, Zhang J.** Autoantibodies to tumor-associated antigens: reporters from the immune system. *Immunol Rev*. 2008;222:328–340.
16. **Kobold S, Luetkens T, Cao Y, Bokemeyer C, Atanackovic D.** Prognostic and diagnostic value of spontaneous tumor-related antibodies. *Clin Dev Immunol*. 2010;2010:721531.
17. **Desmetz C, Mange A, Maudelonde T, Solassol J.** Autoantibody signatures: progress and perspectives for early cancer detection. *J Cell Mol Med*. 2011;15:2013–2024.
18. **Vitale M.** SEREX: a promising approach for identification of thyroid cancer serological biomarkers. *Clin Endocrinol (Oxf)*. 2013;79:12–13.
19. **Klade CS, Voss T, Krystek E, Ahorn H, Zatloukal K, Pummer K, Adolf GR.** Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. *Proteomics*. 2001;1:890–898.
20. **Ghossein R.** Update to the College of American Pathologists reporting on thyroid carcinomas. *Head Neck Pathol*. 2009;3:86–93.
21. **Raparia K, Min SK, Mody DR, Anton R, Amrikachi M.** Clinical outcomes for "suspicious" category in thyroid fine-needle aspiration biopsy: Patient's sex and nodule size are possible predictors of malignancy. *Arch Pathol Lab Med*. 2009;133:787–790.
22. **Banks ND, Kowalski J, Tsai HL, Somervell H, Tufano R, Dackiw AP, Marohn MR, Clark DP, Umbricht CB, Zeiger MA.** A diagnostic predictor model for indeterminate or suspicious thyroid FNA samples. *Thyroid*. 2008;18:933–941.
23. **Kamran SC, Marqusee E, Kim MI, Frates MC, Ritner J, Peters H, Benson CB, Doubilet PM, Cibas ES, Barletta J, Cho N, Gawande A, Ruan D, Moore FD, Jr., Pou K, Larsen PR, Alexander EK.** Thyroid nodule size and prediction of cancer. *J Clin Endocrinol Metab*. 2013;98:564–570.
24. **McCoy KL, Jabbour N, Ogilvie JB, Ohori NP, Carty SE, Yim JH.** The incidence of cancer and rate of false-negative cytology in thyroid nodules greater than or equal to 4 cm in size. *Surgery*. 2007;142:837–844.
25. **Izawa S, Okamura T, Matsuzawa K, Ohkura T, Ohkura H, Ishiguro K, Noh JY, Kamijo K, Yoshida A, Shigemasa C, Kato M, Yamamoto K, Taniguchi S.** Autoantibody against WD repeat domain 1 is a novel serological biomarker for screening of thyroid neoplasia. *Clin Endocrinol (Oxf)*. 2013;79:35–42.
26. **Kiyamova R, Garifulin O, Gryshkova V, Kostianets O, Shyian M, Gout I, Filonenko V.** Preliminary study of thyroid and colon cancers-associated antigens and their cognate autoantibodies as potential cancer biomarkers. *Biomarkers*. 2012;17:362–371.
27. **Abols A, Ducena K, Zayakin P, Silina K, Kalnina Z, Sadovska L, Tars J, Vilmanis J, Narbuts Z, Eglitis J, Pirags V, Line A.** Survey of autoantibody responses against tumor-associated antigens in thyroid cancer. *Cancer Biomark*. 2014;14:361–369.
28. **Maio M, Coral S, Sigalotti L, Elisei R, Romei C, Rossi G, Cortini E, Colizzi F, Fenzi G, Altomonte M, Pinchera A, Vitale M.** Analysis of cancer/testis antigens in sporadic medullary thyroid carcinoma: expression and humoral response to NY-ESO-1. *J Clin Endocrinol Metab*. 2003;88:748–754.
29. **Garg M, Kanojia D, Suri S, Gupta S, Gupta A, Suri A.** Sperm-associated antigen 9: a novel diagnostic marker for thyroid cancer. *J Clin Endocrinol Metab*. 2009;94:4613–4618.
30. **Parmigiani RB, Bettoni F, Vibranovski MD, Lopes MH, Martins WK, Cunha IW, Soares FA, Simpson AJ, de Souza SJ, Camargo AA.** Characterization of a cancer/testis (CT) antigen gene family capable of eliciting humoral response in cancer patients. *Proc Natl Acad Sci U S A*. 2006;103:18066–18071.
31. **Qian-Lin Z, Ting-Feng W, Qi-Feng C, Min-Hua Z, Ai-Guo L.** Inhibition of cytosolic chaperonin CCTzeta-1 expression depletes proliferation of colorectal carcinoma in vitro. *J Surg Oncol*. 2010;102:419–423.
32. **Yue F, Wang LS, Xia L, Wang XL, Feng B, Lu AG, Chen GQ, Zheng MH.** Modulated T-complex protein 1 zeta and peptidyl-prolyl cis-trans isomerase B are two novel indicators for evaluating lymph node metastasis in colorectal cancer: Evidence from proteomics and bioinformatics. *Proteomics Clin Appl*. 2009;3:1225–1235.
33. **Kashyap MK, Harsha HC, Renuse S, Pawar H, Sahasrabuddhe NA, Kim MS, Marimuthu A, Keerthikumar S, Muthusamy B, Kandasamy K, Subbannayya Y, Prasad TS, Mahmood R, Chaerkady R, Meltzer SJ, Kumar RV, Rustgi AK, Pandey A.** SILAC-based quantitative proteomic approach to identify potential biomarkers from the esophageal squamous cell carcinoma secretome. *Cancer Biol Ther*. 2010;10:796–810.
34. **Lin Jf, Xu J, Tian Hy, Gao X, Chen Qx, Gu Q, Xu Gj, Song Jd, Zhao Fk.** Identification of candidate prostate cancer biomarkers in prostate needle biopsy specimens using proteomic analysis. *Int J Cancer* 2007;121:2596–2605.
35. **Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, Chinnaiyan AM.** Large-scale meta-analysis of cancer microarray data identifies common transcriptional profiles of neoplastic transformation and progression. *Proc Natl Acad Sci U S A*. 2004;101:9309–9314.

36. **Roque L, Rodrigues R, Pinto A, Moura-Nunes V, Soares J.** Chromosome imbalances in thyroid follicular neoplasms: a comparison between follicular adenomas and carcinomas. *Genes Chromosomes Cancer*. 2003;36:292–302.
37. **Wreesmann VB, Ghossein RA, Hezel M, Banerjee D, Shaha AR, Tuttle RM, Shah JP, Rao PH, Singh B.** Follicular variant of papillary thyroid carcinoma: genome-wide appraisal of a controversial entity. *Genes Chromosomes Cancer*. 2004;40:355–364.
38. **Lagarkova MA, Koroleva EP, Kuprash DV, Boitchenko VE, Kashkarova UA, Nedospasov SA, Shebzukhov YV.** Evaluation of humoral response to tumor antigens using recombinant expression-based serological mini-arrays (SMARTA). *Immunol Lett*. 2003;85: 71–74.
39. **Scanlan MJ, Welt S, Gordon CM, Chen YT, Gure AO, Stockert E, Jungbluth AA, Ritter G, Jager D, Jager E, Knuth A, Old LJ.** Cancer-related serological recognition of human colon cancer: identification of potential diagnostic and immunotherapeutic targets. *Cancer Res*. 2002;62:4041–4047.
40. **Zhang JY, Casiano CA, Peng XX, Koziol JA, Chan EK, Tan EM.** Enhancement of antibody detection in cancer using panel of recombinant tumor-associated antigens. *Cancer Epidemiol Biomarkers Prev*. 2003;12:136–143.
41. **Bei R, Masuelli L, Palumbo C, Modesti M, Modesti A.** A common repertoire of autoantibodies is shared by cancer and autoimmune disease patients: Inflammation in their induction and impact on tumor growth. *Cancer Lett*. 2009;281:8–23.
42. **Chan J.** Strict criteria should be applied in the diagnosis of encapsulated follicular variant of papillary thyroid carcinoma. *Am J Clin Pathol*. 2002;117:16–18.
43. **Rosai J.** The encapsulated follicular variant of papillary thyroid carcinoma: back to the drawing board. *Endocr Pathol*. 2010;21:7–11.
44. **Kakudo K, Bai Y, Liu Z, Ozaki T.** Encapsulated papillary thyroid carcinoma, follicular variant: a misnomer. *Pathol Int*. 2012;62:155–160.
45. **Kakudo K, Bai Y, Liu Z, Li Y, Ito Y, Ozaki T.** Classification of thyroid follicular cell tumors: with special reference to borderline lesions. *Endocr J*. 2012;59:1–12.
46. **Baloch ZW, Livolsi VA.** Encapsulated follicular variant of papillary thyroid carcinoma with bone metastases. *Mod Pathol*. 2000;13: 861–865.
47. **Goldstein NS, Czako P, Neill JS.** Metastatic minimally invasive (encapsulated) follicular and Hurthle cell thyroid carcinoma: a study of 34 patients. *Mod Pathol*. 2000;13:123–130.
48. **Terada T.** Brain metastasis from thyroid adenomatous nodules or an encapsulated thyroid follicular tumor without capsular and vascular invasion: a case report. *Cases J*. 2009;2:7180.
49. **Williams ED.** Guest Editorial: Two Proposals Regarding the Terminology of Thyroid Tumors. *Int J Surg Pathol*. 2000;8:181–183.