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An antibody-like peptide that recognizes malignancy among thyroid nodules

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ABSTRACT

There is an urgent need for biomarkers to identify malignant thyroid nodules from indeterminate follicular lesions. We have used a subtractive proteomic strategy to identify novel biomarkers by selecting ligands to goiter tissue from a 12-mer random peptide phage-displayed library using the BRASIL method (Biopanning and Rapid Analysis of Selective Interactive Ligands). After three rounds of selection, two highly reactive clones to the papillary thyroid tumor cell line NPA were further evaluated, and their specific binding to tumor proteins was confirmed using phage-ELISA. The antibody-like peptide CaT12 was tumor-specific, which was further tested by immunohistochemistry against TMAs (tissue microarrays) comprised of 775 human benign and malignant tissues, including 232 thyroid nodular lesions: 15 normal thyroid tissues, 53 nodular goiters (NG), 54 follicular adenomas (FA); 69 papillary thyroid carcinomas (PTC); and 41 follicular carcinomas (FC). CaT12 was able to identify PTC among thyroid nodular lesions with 91.2% sensitivity and 85.1% specificity, despite its non-specificity for thyroid tissues. Additionally, the CaT12 peptide helped characterize follicular lesions distinguishing the follicular variant of PTC (FVPTC) from FA with 91.9% accuracy; FVPTC from NG with 83.1% accuracy; FVPTC from the classic PTC with 57.7% accuracy; and FVPTC from FC with 88.7% accuracy. In conclusion, our strategy to select differentially expressed ligands to thyroid tissue was highly effective and resulted in a useful antibody-like biomarker that recognizes malignancy among thyroid nodules and may help distinguish follicular patterned lesions.

1. Introduction

Thyroid cancer incidence rates have steadily increased over the past few decades all over the world [1] and 56,460 new patients are estimated to be diagnosed during 2012 in the USA [2]. The accessible use of sensitive imaging detection methods is mainly responsible for this increased incidence [3]. However, there are evidences that other factors, including the exposure to environmental carcinogenic factors, may have also contributed to this phenomenon [3,4]. We have actually experienced real epidemics of thyroid nodules referred to specialized diagnosis, and the cytological exam of fine-needle cell aspiration is the recommended method by current guidelines to distinguish benign from malignant lesions [5].

In most cases, the clinical features of the patients based on ultrasonography and cytological findings enable appropriate case management [5]. However, up to one third of the nodules submitted to fine needle aspiration biopsies may present an "indeterminate" cytology, a pattern that remains burdened with inter-observer variability and uncertainty regarding management [6]. Even the most experienced pathologists may have difficulties distinguishing cases of follicular variants of papillary thyroid carcinomas from simple benign hyperplasia, and many cases of follicular adenomas are submitted to unnecessary surgeries because it is virtually impossible to eliminate the risk of a follicular carcinoma in cytological analyses [7]. A series of molecular markers for malignancy have been proposed and many of them appear to be effective in reducing uncertainties [8,9]. However, clinical problems concerning diagnosis of thyroid malignancies are not solved, and certainly are not appropriate to large populations.

In the present investigation, we have used a different approach to distinguish thyroid malignancies. We employed phage display

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technology that has been successfully used to select targets for various diseases [10–12], including many cancer types [13–24], and, by using a direct selection (BRASIL) against goiter tissue, we were able to obtain a highly reactive and discrimination marker for thyroid cancer diagnosis.

2. Materials and methods

Research Ethics Committees of three institutions approved this study: Federal University of Uberlândia (UFU), State University of Campinas (UNICAMP) and Fundação Antônio Prudente (Hospital AC Camargo, SP). All participants signed informed consent forms.

2.1. Cell culture and patients' samples

We have used the TPC-1 (Human thyroid papillary cancer) and NPA (Human papillary cancer) cells, gently provided by Prof. J.M. Cerutti, from the Federal University of São Paulo. In addition, we have developed a primary human cell culture using tissue obtained from a patient submitted to surgical removal of a multinodular goiter at the Surgery Center of UFU Hospital. Thyroid cancer cell lines and goiter-derived cells were grown in RPMI 1640 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum (Nutricell, Campinas-SP) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Fresh tissues from patients with histologically-proved classic PTC and hyperplasia were obtained from the Federal University of Uberlândia (UFU) Clinics' Hospital, and immediately frozen until use for protein binding testing.

The selected clone was tested by immunohistochemistry in formalin fixed, paraffin embedded tissues from 775 patients reviewed for diagnostic confirmation in order to select the most representative areas designed to build tissue microarrays (TMA, Beecher Instruments®, Silver Springs, MD, USA). A first TMA was composed by 232 patient samples: 15 normal thyroid tissues, 53 nodular goiters, 54 follicular adenomas, 69 papillary thyroid carcinomas (36 cases of the classical form and 33 of the PTC follicular variant) and 41 follicular carcinomas. A second TMA was designed to validate our results, and was constituted by 281 other cancer tissues (189 breast, 57 prostate and 35 kidney), 41 benign lesions (12 nevus atypical and 29 nevus) and 221 non-neoplastic tissues (including normal stomach, ovary, breast, prostate, kidney, heart, muscle, brain, liver, lung and others).

Patients' clinical information was obtained from their clinical records. Aggressiveness at diagnosis was ascertained using the Tumor Node Metastasis and stage classification systems for DTC as recommended by the American Thyroid Association guidelines [5]. For statistical purpose we grouped patients classified as stage II, III and IV in one class named "higher stages". Patients were followed with periodic total body scans, serum TSH and thyroglobulin (Tg) measurements, according to a standard protocol that included X-ray, ultrasonography, computed tomography scan and other eventual procedures to detect distant metastasis for a period of 12–298 months (43.50 ± 33.29 months). Patients presenting high non-stimulated serum Tg levels (>2 mg/dl) were submitted to image evaluation. We defined tumors as persistent/recurrent and/or presenting long distance metastasis, according to the aforementioned parameters.

2.2. Biopanning in cell surface

Biopanning was performed in three rounds using BRASIL method (Biopanning and Rapid Analysis of Selective Interactive Interactive Ligands) [25] to verify phage binding to intact cells.

2.3. DNA sequencing of selected phage clones

After three rounds, 96 blue colonies were randomly selected and their phage single-strand DNA were isolated by the iodide buffer extraction procedure (Instruction Manual Ph.D.-12 Phage Display Peptide Library Kit) and analyzed with MegaBace 1000 Genetic Analyzer (Amersham Biosciences) automatic capillary sequencer. The nucleotide sequence of the gene III insert was sequenced with −96 gIII sequencing primer 5′-CCC TCA TAG TTA GCG TAA CG-3′ by automated dye terminator cycle sequencing. The amino acid sequence of the insert was deduced from the nucleotide sequence using DNA2PRO software from RELIC program [26,27].

2.4. ELISA (enzyme-linked immunosorbent assay)

After extraction of papillary cancer and goiter proteins with extraction buffer (Tris–HCl 20 mM pH7.2, EDTA 10 mM, EGTA 2 mM, sucrose 250 mM, DTT 1 mM, Benzamidine 1 mM) proteins were diluted in sodium carbonate buffer (10 μg/ml in 0.1 M $NaHCO_3$) and immobilized into a 96-wells plate (Nunc Immuno Maxisorp, Roche Diagnostics) by incubation it overnight at 4 °C. That plate was subsequently washed three times with PBST 0.1%, and blocked with BSA 3% (blocking buffer) for three hours at 37 °C. Plate wells were washed again three times, and each well was incubated with 100 μl of phages supernatants for one hour at 37 °C. After five washes, recombinant phage particles were detected with peroxidase-conjugated anti-M13 monoclonal antibody (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) using a 1:5000 dilution in blocking buffer. The peroxidase staining reaction was developed in the presence of SIGMA*FAST*™ OPD (*o*-phenylenediamine dihydrochloride) tablets (Sigma–Aldrich, São Paulo, SP, Brazil), and then incubated at room temperature for 5 min, stopped with diluted acid solutions, and the optical density (OD) values were measured on a microplate reader (TP-READER – Thermo plate) at 492 nm.

2.5. Recombinant phage and synthetic peptide immunohistochemistry (IHC)

After deparaffinizing the tissues, the endogenous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide for three times, 5 min each. The tissue sections were then rinsed thoroughly in distilled water. For the biomarker exposure, sodium citrate buffer (0.1 M) was heated to 95 °C steamer, the slides were submerged into the buffer for 35 min, washed for 5 min in running water, and blocked with 3% skim milk (Molico Milk, Nestle, Brazil; 3% w/v in distilled water) for 30 min at room temperature. Incubation with the primary marker (phage or synthetic peptide) was then performed for 30 min at 37 °C and overnight at 4 °C. The recombinant phage CaT12, the synthetic peptide (Invitrogen, Carlsbad, CA, USA), and the wild phage (no peptide) were diluted into 100 μl of the Novocastra Universal IHC Blocking/Diluent 1000 (Leica Microsystems Inc, Buffalo Grove, IL, USA) containing 6×10^{11} pfu (viral particles/slide) or 1 μg synthetic peptide. As negative controls, duplicate sections were incubated with 3% skim milk proteins instead of specific primary antibodies. The sections were washed four times in PBS for 5 min each followed by one hour incubation with peroxidase-conjugated mouse anti-M13 secondary antibody or streptavidin. After a washing step in PBS, peroxidase activity was visualized by incubation in 3,3′diaminobenzidine tetrahydrochloride (Liquid DAB, Sigma, St. Louis, USA) for 5 min at room temperature. Sections were counterstained with hematoxylin. Positive and negative controls were run in the same reaction batch. Slides were independently scored by two experienced researchers (CFR and ECM), both blinded to tumor features, and confirmed by an experienced pathologist (JV).

2.6. Immunohistochemistry staining index

Signals were considered positive when reaction products were localized in the expected cellular component. The staining index value was obtained by the sum of intensity and distribution scores, and values of a staining index equal or greater than 5 were considered positive.

The criteria employed for the intensity score were: 0, no staining; 1, weak; 2, moderate; 3, strong. The criteria employed for the distribution score were: 0, no staining; 1, staining of <25% of the cells; 2, between 25% and 50% of the cells; 3, between 50% and 75% of the cells; and 4, staining of >75% of the cells.

In addition, we employed the Automated Cellular Imaging System ACIS-III (Chroma Vision Medical Systems, Inc, DAKO, San Juan Capistrano, CA, USA) for IHC quantification. Briefly, each tissue spot was digitalized to the systems' software, and a computer algorithm, considering the intensity and extension of the brown staining, attributed numerical values.

2.7. Streptavidin-bead precipitation assay

TPC-1 cells were lysed according to the instructions of the RIPA Buffer (Thermo scientific, Rockford, IL, USA) kit; a protease inhibitor cocktail (1:100 dilution) (Sigma–Aldrich, São Paulo, SP, Brazil) was added and the protein concentration was determined using the BCA Protein Assay Kit (Thermo scientific). The synthetic peptide (1 mg/ml) was N-terminal labeled with biotin, and mixed with 200 μl packed Streptavidin-beads (Thermo scientific) for 1 h at 4 °C, and incubated with 500 μg of protein extracts for 4 h at room temperature. After washing four times with TBS (Tris Buffered Saline), the proteins were eluted from the beads by addition 0.1 M glycine HCl buffer, pH 3.0, twice for 10 min each. The eluate was quantified by the BCA Protein Assay Kit prior to proteins sequencing.

2.8. Protein sequencing by LC-MS/MS and target identification

Eluted proteins were precipitated out of solution using the ProteoExtract kit (Calbiochem) and the protein pellet was left to dry overnight in a sterile fumehood. The lyophilized pellet was then resuspended in 50 mM ammonium bicarbonate (pH 8.0) and subjected to an in-solution tryptic digestion (Mike Myers, Cold Spring Harbor modified by Brett S. Phinney, UC Davis Proteomics Core, Davis, CA, USA). Digested peptides were then de-salted using aspire tips (Thermo-Fisher Scientific, RP30 tips) before being resuspended in loading buffer.

Digested peptides were analyzed using a LTQ-FT (Thermo Fisher Scientific) coupled with a MG4 paradigm HPLC (Michrom, Auburn, CA, USA). The samples were loaded onto a Michrom cap trap (0.5 × 2 mm) to be de-salted online. The peptides were then separated using a Michrom Magic C18AQ (200 μm × 150 mm) reversed-phase column and eluted using a gradient for 60 min. Collision induced dissociation was applied to the peptide samples and data was acquired with an isolation width of 1, normalized collision energy of 35, and a resolution of 50,000. The spray voltage on the Michrom captive spray was set to 1.8 kV with a heated transfer capillary temperature of 200 °C.

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