

Keywords: thyroid cancer; phage display; biomarker; scFv antibody

A putative OTU domain-containing protein 1 deubiquitinating enzyme is differentially expressed in thyroid cancer and identifies less-aggressive tumours

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Background: This study aimed to identify novel biomarkers for thyroid carcinoma diagnosis and prognosis.

Methods: We have constructed a human single-chain variable fragment (scFv) antibody library that was selected against tumour thyroid cells using the BRASIL method (biopanning and rapid analysis of selective interactive ligands) and phage display technology.

Results: One highly reactive clone, scFv-C1, with specific binding to papillary thyroid tumour proteins was confirmed by ELISA, which was further tested against a tissue microarray that comprised of 229 thyroid tissues, including: 110 carcinomas (38 papillary thyroid carcinomas (PTCs), 42 follicular carcinomas, 30 follicular variants of PTC), 18 normal thyroid tissues, 49 nodular goitres (NG) and 52 follicular adenomas. The scFv-C1 was able to distinguish carcinomas from benign lesions ($P=0.0001$) and reacted preferentially against T1 and T2 tumour stages ($P=0.0108$). We have further identified an OTU domain-containing protein 1, DUBA-7 deubiquitinating enzyme as the scFv-binding antigen using two-dimensional polyacrylamide gel electrophoresis and mass spectrometry.

Conclusions: The strategy of screening and identifying a cell-surface-binding antibody against thyroid tissues was highly effective and resulted in a useful biomarker that recognises malignancy among thyroid nodules and may help identify lower-risk cases that can benefit from less-aggressive management.

The rapid development and widespread use of sensitive imaging techniques, such as the ultrasonography, has resulted in increased frequency of clinically silent lesions that are submitted to diagnostic aspirative cytology (Guth *et al*, 2009).

A parallel increase in the number of patients diagnosed with papillary thyroid carcinomas (PTCs) has been registered almost all

over the world (Kilfoy *et al*, 2009). Nevertheless, mortality rates have remained stable (Davies and Welch, 2010), suggesting that part of these lesions would possibly never evolve to clinically significant cancers. In fact, there is increasing evidence indicating that some immunologic features of the tumour and/or its host may restrain the thyroid cancer development in some individuals

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Received 24 December 2013; revised 20 March 2014; accepted 15 May 2014; published online 17 June 2014

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(Cunha *et al*, 2012a,b). Conversely, a considerable number of cytological analyses resulted as indeterminate, leading up to one-third of patients with thyroid nodules to surgeries that were later proven unnecessary, as final histology demonstrated benignancy in 70–80% of the indeterminate citologies (Piana *et al*, 2011). This troubling scenario urges the immediate search for reliable diagnostic markers.

Monoclonal antibodies have been used for antigen detection and therapeutics, and their specificity combined with low toxicity make them a promising pharmaceutical commodity. In fact, after vaccines, they comprise the second-largest category of biological medicines in clinical development (Berger *et al*, 2002). Successful examples include rituximab, approved by the FDA since 1997, an anti-CD20 chimeric antibody that became an integral component of many treatment strategies for non-Hodgkin's lymphoma (Seymour, 2004), and OKT3, an anti-CD3 that is used to reduce graft rejection (Cosimi *et al*, 1981).

In the present investigation, we have used the combinatorial single-chain variable fragment (scFv) antibody library displayed on a filamentous phage capsid to select and identify tumour-specific antigens in thyroid carcinoma cell surfaces. A highly reactive marker was able to distinguish thyroid malignancies, and may also be useful to identify lower-risk cases.

MATERIALS AND METHODS

The Research Ethics Committees from University of Campinas (Unicamp), Federal University of Uberlandia (UFU), and Fundação Antônio Prudente (Hospital A. C. Camargo) approved this study under the protocol numbers 096/2008, 026/2009 and 1259/2009, respectively, and a written informed consent was obtained from all patients.

Cell cultures and patients. We used a PTC cell line, NPA, kindly provided by Professor JM Cerutti, from the Federal University of São Paulo, and we also developed a goitre primary human cell culture using fresh thyroid tissue obtained from a patient submitted to surgical removal of a multinodular goitre at the Surgery Center of the UFU Hospital.

Two hundred and twenty-nine thyroid cases consecutively examined at the Hospital A. C. Camargo were assembled into a tissue microarray (TMA) that included 110 samples of well-differentiated thyroid carcinomas (38 classic forms of papillary thyroid cancer; 30 follicular variants of papillary thyroid cancers and 42 follicular thyroid cancers); 52 follicular adenomas; 49 nodular goitres and 18 normal thyroid tissues. Aggressiveness at diagnosis was ascertained using the American Joint Committee on Cancer/International Union against Cancer staging system, which is based on the tumour size, extrathyroidal invasion, lymph node and distant metastases (TNM), and age; and risk stratification according to the Brazilian updated consensus on thyroid nodules and differentiated thyroid cancer (Rosario *et al*, 2013). Using these parameters, the patients were grouped into lower-risk (including very low- and low-risk patients) and higher-risk (intermediate and high risk) cases. Patients were followed with periodic whole-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) (Rosario *et al*, 2013). Patients presenting high nonstimulated serum Tg levels (> 1 ng dl⁻¹) were submitted to image evaluation. We defined tumours as persistent/recurrent and/or presenting long-distance metastasis, according to the aforementioned parameters.

Construction of human scFv phage library. A scFv antibody library was constructed from total RNA samples obtained from

leukocyte cells (peripheral blood) of 25 patients with well-differentiated thyroid carcinoma. RNA was extracted with TRIzol reagent and then they were mixed together. After that, 20 µg of the mixture were submitted to cDNA synthesis with the Superscript III kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Afterwards, heavy- and light-chain variable regions (V_H and V_L) were separately amplified by PCR using primers described elsewhere (Marks *et al*, 1991). All primers used in this study are listed in Supplementary Table 1.

Amplification reactions consisted of 10 µl 10 × PCR buffer, 60 pmol of each primer, 0.25 U of Taq enzyme, 5.0 µl MgCl₂ (50 mM), 10 µl deoxyribonucleotide mix (2 mM), 10 µl cDNA product and water to reach a final volume of 100 µl. PCR conditions were: 94 °C for 5 min, 37 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1.5 min. The amplified V_H and V_L segments were purified from the agarose gel after electrophoresis, pooled and linked through a series of overlap PCR steps to give the final scFv products in V_L-linker-V_H format, with GGSSRSS linker (Andris-Widhopf *et al*, 2011).

The overlapping PCR reaction consisted of 37 cycles of 94 °C for 5 min, 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1.5 min, followed by a final extension at 72 °C for 10 min. The scFv gene products were purified and ligated into the display vector pComb3X, both digested with *Sfi*I restriction enzyme, followed by electroporation into XL1-Blue (Stratagene, La Jolla, CA, USA) electrocompetent cells. Colonies were grown on agar plates with 100 µg ml⁻¹ carbenicillin and 40 mM glucose. Afterwards, the colonies were counted to determine the library size, as described elsewhere (Andris-Widhopf *et al*, 2000). The scFv antibody fragments displayed on the surface of the filamentous phage was performed by rescuing with VCS-M13 helper phage (Stratagene), as previously described (Rader and Barbas, 1997).

DNA sequencing analysis. Single-strand DNA was isolated, and the heavy- and light-chain variable region genes were sequenced using MegaBACE 1000 automatic sequencer (Molecular Dynamics, Sunnyvale, CA, USA). The sequencing reaction was carried out using the DyEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare, USA), following the manufacturer's instructions. The nucleotide sequence of the V_H and V_L was determined using MMB4 (5'-GCTTCCGGCTCGTATGTTGTGT-3') and MMB5 (5'-CGTTTGCCATCTTTTCATAATC-3') oligonucleotides, and immunoglobulin variable domain sequences and V gene families were analysed using the Ig-BLAST program at NCBI (<http://www.ncbi.nlm.nih.gov>) and VBASE2 (<http://www.vbase2.org>) (Retter *et al*, 2005).

Selection of scFv library on cells. Three selection cycles were performed using the phage display technology and the BRASIL method (biopanning and rapid analysis of selective interactive ligands) (Giordano *et al*, 2001) to verify phage binding to intact cells. Briefly, the phage displayed scFv library was positively selected by biopanning on PTC cell line (NPA) after a previous negative selection against goitre primary human cell culture (benign disease) to eliminate major cross-reactivities. Cells were collected in DMEM at 1 × 10⁵ cells ml⁻¹ and incubated with phage in 1.5-ml microtubes for 1 h on ice. Subsequently, 100 µl of the cell-phage suspension was transferred to a new tube containing 200 µl of dibutylphthalate:cyclohexane (9:1; v-v) and centrifuged at 10 000 g for 10 min at 25 °C. Alternatively, in the steps of negative selection when phages were incubated with the strain of benign thyroid cells after centrifugation, phages were collected above the liquid phase from the organic layer and these phages were incubated for an additional 1.5 h with tumour cells on ice. Finally, the tube was frozen in liquid nitrogen, the bottom of the tube sliced off and the cell-phage pellet transferred to a tube containing 50 ml culture of *E. coli* XL1-Blue in log phase and incubated for 15 min at room temperature to phage recovery.

Expression of soluble scFvs in *E. coli*. After the third round selection, plasmidial DNA from XL1-Blue cells was extracted and used to transform electrocompetent non-suppressor TOP10 cells (One ShotTOP10 Electrocomp *E. coli*, Invitrogen) for soluble expression of scFv molecules. Ninety-six random clones were chosen from plates and inoculated into a 2-ml deep-well plate (NUNC, Roskilde, Germany) in 1 ml of super broth (SB) medium containing $100 \mu\text{g ml}^{-1}$ carbenicillin and 40 mM glucose, then followed by incubation for 14 h at 37°C and shaken at 300 r.p.m. After amplification, $10 \mu\text{l}$ of cells were transferred to a new deep-well plate containing the same medium and incubated for 4 h at 37°C and 300 r.p.m., afterwards followed by centrifugation at 3000 g for 10 min. The supernatants were substituted by 1 ml of SB medium containing $100 \mu\text{g ml}^{-1}$ carbenicillin and 2.5 mM of isopropyl- β -D-thiogalactoside (IPTG, Sigma, St Louis, MO, USA); after that, the scFv induction was carried out for 18 h at 30°C and 300 r.p.m. The supernatant containing the scFv was obtained by 3000g centrifugation for 20 min and was directly subjected to dot-blot immunoassay and purification.

Soluble scFv antibody production and purification. The scFv clones were transformed in TOP10 *E. coli* non-suppressor strain and then used to inoculate SB medium containing $50 \mu\text{g ml}^{-1}$ of carbenicillin and 2% glucose. The culture was grown under agitation (250 r.p.m.) overnight at 37°C and 2 ml were diluted in 200 ml of supplemented SB medium. The culture was incubated under agitation for 6–8 h at 37°C ($\text{OD}_{600} = 1.0$), and centrifuged at 3000 g for 10 min at 4°C . Bacteria were resuspended in 200 ml supplemented carbenicillin SB medium and induced by 2 mM IPTG at 30°C overnight. Supernatant containing antibody was obtained after centrifugation at 5000 g, 4°C , 15 min, after that, His-tagged scFv fragments were purified by immobilized-metal (Ni) affinity chromatography (HisTrap HP, GE Healthcare) by high-performance liquid chromatography (HPLC, Amersham Biosciences, Sunnyvale, CA, USA) according to the manufacturer's instructions. All eluted fractions were identified for the presence of antibody fragments by dot-blot immunoassay, positive fractions were pooled, desalted by a Centriprep column (Millipore, Billerica, MA, USA), lyophilised, resuspended in PBS and then measured at 280 nm for protein. The purity and correct size of scFv was verified on 12% SDS-PAGE gel followed by silver staining (ProteoSilver Plus Silver Stain Kit, Sigma).

ELISA screening. Maxisorp 96-well (NUNC) plates were coated with $1.0 \mu\text{g}$ per well of total pooled protein extracted from goitre, adenoma and tumour thyroid tissues to screen for clones that could differentially recognise thyroid cancer antigens in $100 \mu\text{l}$ of sodium bicarbonate buffer (pH 7.4) overnight at 4°C . Wells were washed three times with PBST and then blocked for 1 h at 37°C with PBS plus 5% nonfat milk (PBSM 5%). After blocking, wells were washed three times with PBST and $4 \mu\text{g}$ of purified scFvs were added to each well and incubated for 2 h at room temperature under low agitation. Then, the plate was washed three times with PBST, followed by incubation with HRP-conjugated rat anti-HA (Roche Applied Science, Indianapolis, IN, USA) diluted (1:1000) in PBSM 5% for 1 h at 37°C . After another four washes, the chromogenic substrate OPD SigmaFast (Sigma-Aldrich) was added and the absorbance was measured at 492 nm.

Tissue microarray (TMA) construction and immunohistochemistry. The affinity of the monoclonal scFv antibody was verified by immunohistochemistry on the TMA. Paraffin-embedded tissue sections from 229 patients were deparaffinized, rehydrated and incubated three times with 3% H_2O_2 for 5 min. The tissue sections were rinsed in distilled water, and then submitted to heat pretreatment in 0.1 M sodium citrate buffer for 35 min at 95°C for antigen retrieval. The sections were rinsed again and then blocked for 1 h in PBS–10% BSA. Then, $4 \mu\text{g ml}^{-1}$ of the scFvs

diluted in Novocastra IHC Diluent (ref. RE7133, Leica Biosystems, Newcastle, United Kingdom) were applied and incubated for 14 h at 4°C overnight. After that, slides were rinsed four times, 5 min each, with PBS. Subsequently, they were incubated with HRP-conjugated rat anti-HA (Roche Applied Science) diluted (1:200) in buffer for 1 h at room temperature, rinsed with PBS and then incubated with Advance HRP Enzyme Dako (San Juan Capistrano, CA, USA) for 30 min at 37°C . The bound antibody was detected with the DAB substrate (Sigma) and counterstained with haematoxylin. Analyses of staining were carried out using the Automated Cellular Imaging System ACIS-III (Chroma Vision Medical Systems Inc, Dako) for IHC quantification.

Briefly, each tissue spot was digitalised to the system software, and a computer algorithm based on the intensity and extension of the brown staining attributed numerical values to each spot. A final score, given by the mean of triplicates, was used for statistical comparisons.

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

Proteins in PTC and goitre tissues were resolved by two-dimensional gel electrophoresis (2D) (Roessler *et al*, 2006). Two gels were performed for both the papillary carcinoma and goitre proteins. One was stained with Coomassie Brilliant Blue G-250 dye (Bio-Rad, Hercules, CA, USA) and the other was transferred onto a nitrocellulose membrane (GE) for western blot with scFv-C1 antibody, using the HRP-conjugated rat anti-HA (Roche Applied Science). The corresponding spots that scFv antibody recognised in the western blot were excised from the gels and subjected to trypsin digestion (Wilm *et al*, 1996), followed by liquid chromatography mass spectrometry (LC-MS/MS) to allow identification.

Peptides were identified by on-line chromatography using a Cap-LC coupled to a Q-TOF Ultima API mass spectrometer (Waters, Milford, MA, USA). The resulting fragmented spectra were processed using the ProteinLynx 4.0 software (Waters), and

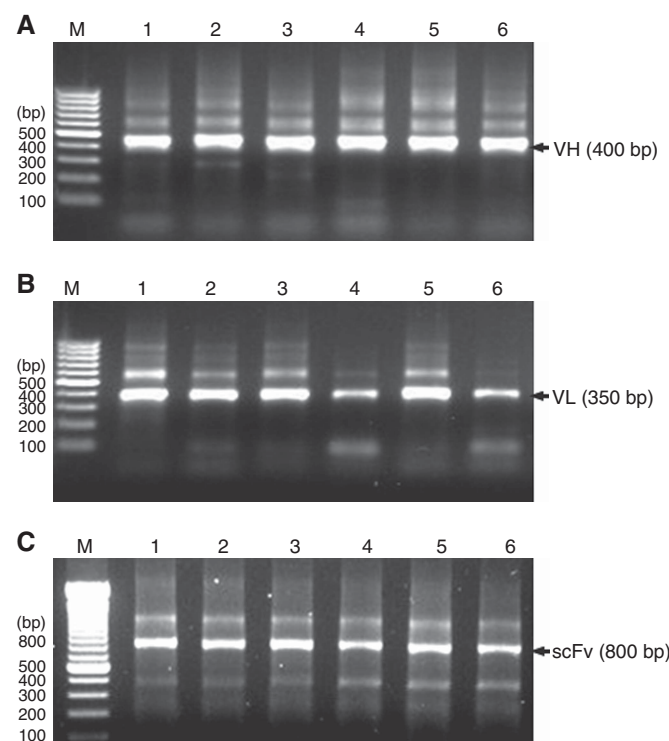


Figure 1. Phage scFv library antibody construction. The amplified gene fragments for the variable regions of the heavy- (V_H) (A) and light-chain (V_L) (B) gene repertoires corresponded to the expected sizes of ~ 400 and ~ 350 bp, respectively. The resulting V_H and V_L fragments were assembled into scFv inserts by overlapping PCR (C).

we used the MASCOT MS/MS Ion Search system to blast the sequences against the SwissProt and NCBI nr databases. According to the MASCOT probability method, only significant ($P<0.05$) hits were accepted.

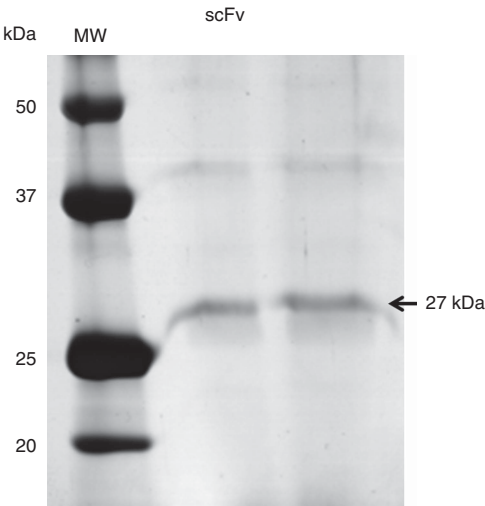


Figure 2. Evaluation of scFv purification after IPTG induction. Soluble scFv antibody was produced by induction of scFv phagemid pComb3X in *E. coli* TOP10 cells. His-tagged scFv fragments were purified by immobilized-metal (Ni) affinity chromatography and subjected to 12% SDS–PAGE. The gel was silver stained using ProteoSilver. A band of the expected size for scFv is 27 kDa. MW, protein marker.

Statistical analysis. We employed GraphPrism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) to analyse the differences of scFv–C1-positive staining in pathological groups. We have used sensitivity, specificity, positive-predictive value and negative-predictive parameters in the Fisher’s test. For ACIS imaging values, the Mann–Whitney test (nonparametric analyses) was applied to determine staining intensity differences between disease groups in the TMA. A value of $P<0.05$ was considered statistically significant.

RESULTS

Phage library construction and characterisation. The amplified gene fragments for the variable regions of the heavy- (V_H) and light-chain (V_L) gene repertoire corresponded to the expected sizes of ~ 400 and ~ 350 bp, respectively (Figure 1A and B). The resulting V_H and V_L fragments were assembled into scFv inserts by overlapped PCR reactions, allowing the random association of heavy and light chains to maximise combinatorial reassortment, as demonstrated in Figure 1C. The resulting library size was 1.02×10^6 clones. Aiming to assess the diversity of scFvs, 48 clones were randomly selected and their DNA sequenced. The variable regions were derived from nine different V gene families, including three V_H gene families (V_H1 , 3 and 4) and six V_L subgroups (V_L1 , 2 and 3 and V_L4 , 5 and 6), demonstrating that the scFv gene fragments were distributed across the full repertoire of antibody germline genes.

The final product containing purified scFv was subjected to SDS–PAGE to identify the purity and molecular mass, as shown in Figure 2. The purified scFv exhibited the protein band size of 27 kDa.

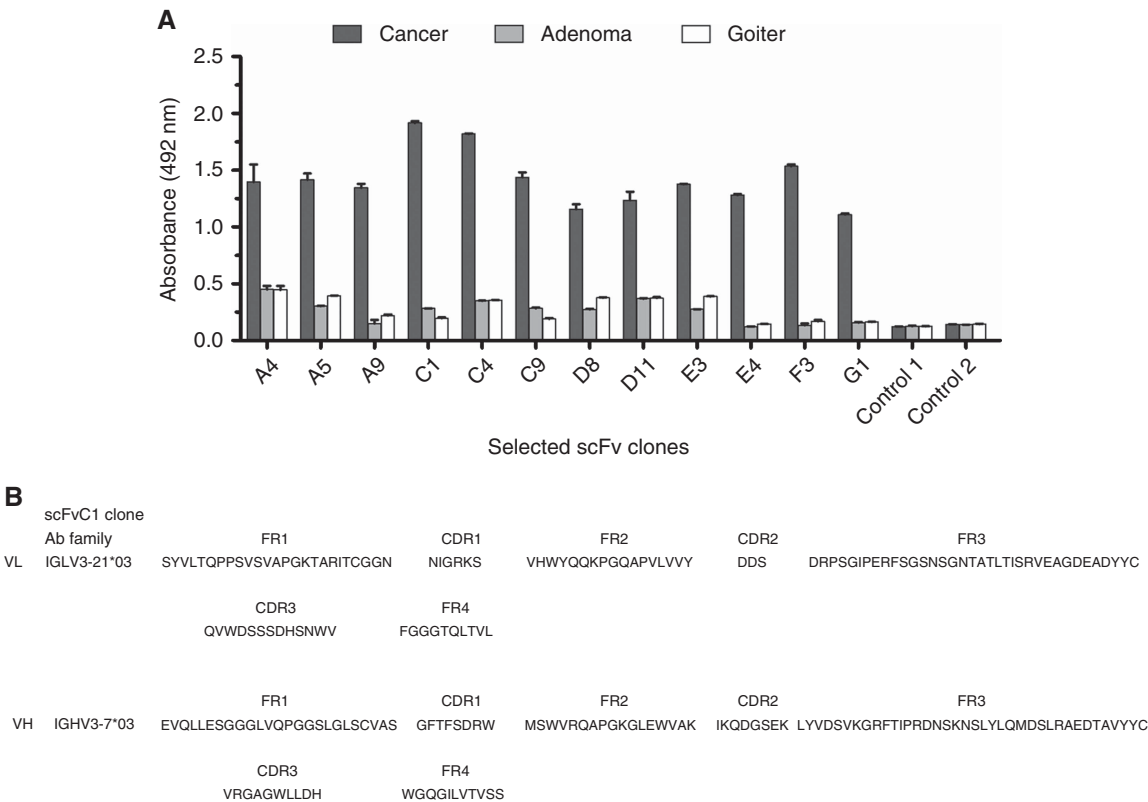


Figure 3. ELISA screening of antibody fragments (scFv). The scFv clones were subjected against a total protein extracted from papillary thyroid cancer (black), follicular adenoma (gray) and goitre (white) tissues. All clones showed better reaction with cancer proteins. No positive signal was observed in the negative control 1 (scFv specific to *Mycobacterium tuberculosis*) and control 2 (pComb3X without insert) scFv–C1 clone was selected for additional procedures (A). The amino-acid sequence of scFv–C1 clone with appropriate regions for framework and CDR residues, variable light-chain (V_L) and the variable heavy-chain (V_H) domains (B).

Dot-blot, ELISA immunoassays validation and DNA sequencing.

In the last round of the screening process, clones were grown in 96 deep-well plates, and soluble expressions of scFv fragments were induced and verified by dot blot. scFv clones with high expression were purified by HPLC, and the specificity of these scFvs against thyroid cancer proteins was determined by ELISA screening. Twelve clones demonstrated reactivity to thyroid proteins, presenting low reactivity to goitre and adenoma proteins, as demonstrated in Figure 3A. Among these clones, the clone named scFv-C1 was selected based on its reactivity to proceed with validation tests. No positive signal was observed in the negative controls (irrelevant scFv, specific to *Mycobacterium tuberculosis* and pComb3X without insert).

The complete nucleotide sequence and predicted amino-acid sequence of the heavy- and light-chain variable region of scFv-C1 clone is shown in Figure 3B, and complete sequencing analysis in Supplementary File 2.

Immunohistochemistry. The scFv monoclonal antibody immunoreactivity was tested on the 229 thyroid samples included in the TMA. Positive staining was observed in PTC, follicular variant of papillary and follicular carcinoma, as demonstrated in Figure 4 panels A, B and C, respectively. Lower positivity was observed among follicular adenomas (28.8%) and goitres (6.1%), whereas all normal thyroids tissues were negative (Figure 4D). No labelling was detected in adjacent normal cells, negative control, scFv with

specificity for *Mycobacterium tuberculosis*, and control tissues without scFv-C1, secondary Ab alone (Figure 4E and F).

The staining area was larger in carcinomas than in controls. The scFv-C1 antibody distinguished thyroid carcinomas from goitres and normal thyroid tissues, as demonstrated in Figure 4G; Table 1. However, this scFv antibody did not differentiate papillary, follicular and follicular variant papillary carcinoma cases. scFv-C1 was able to differentiate lower-risk from higher-risk ($P=0.0108$) patients. Also, the ACIS immunostaining score was higher in the lower-risk group, as shown in Figure 4H; Table 2.

Identification of OTUD1 deubiquitinating enzyme in thyroid cancer.

The 2D gel electrophoresis method was performed to separate the proteins extracted from a pool of the PTC cases and the goitres. Figure 5 shows the representative proteomic profile for papillary thyroid carcinoma, in pannel A, in comparison with goitre tissues in panel B. Figure 5 represents the results obtained by the incubation of scFv-C1 antibody with thyroid carcinoma, in panel C, and with goitre in panel D. The scFv antibody recognised two spots; spot 1 was specifically detected in cancer, whereas spot 2 was commonly detected in both cancer and goitre. Spot 1 was further identified as the OTU domain-containing protein 1, a deubiquitinating enzyme, and the spot 2 was identified as haemoglobin subunit alpha, explaining the cross-reactivity with the secondary antibody. The theoretical molecular mass/pI value for OTU1-domain was 51.063 kDa/5.68, fitting well with the

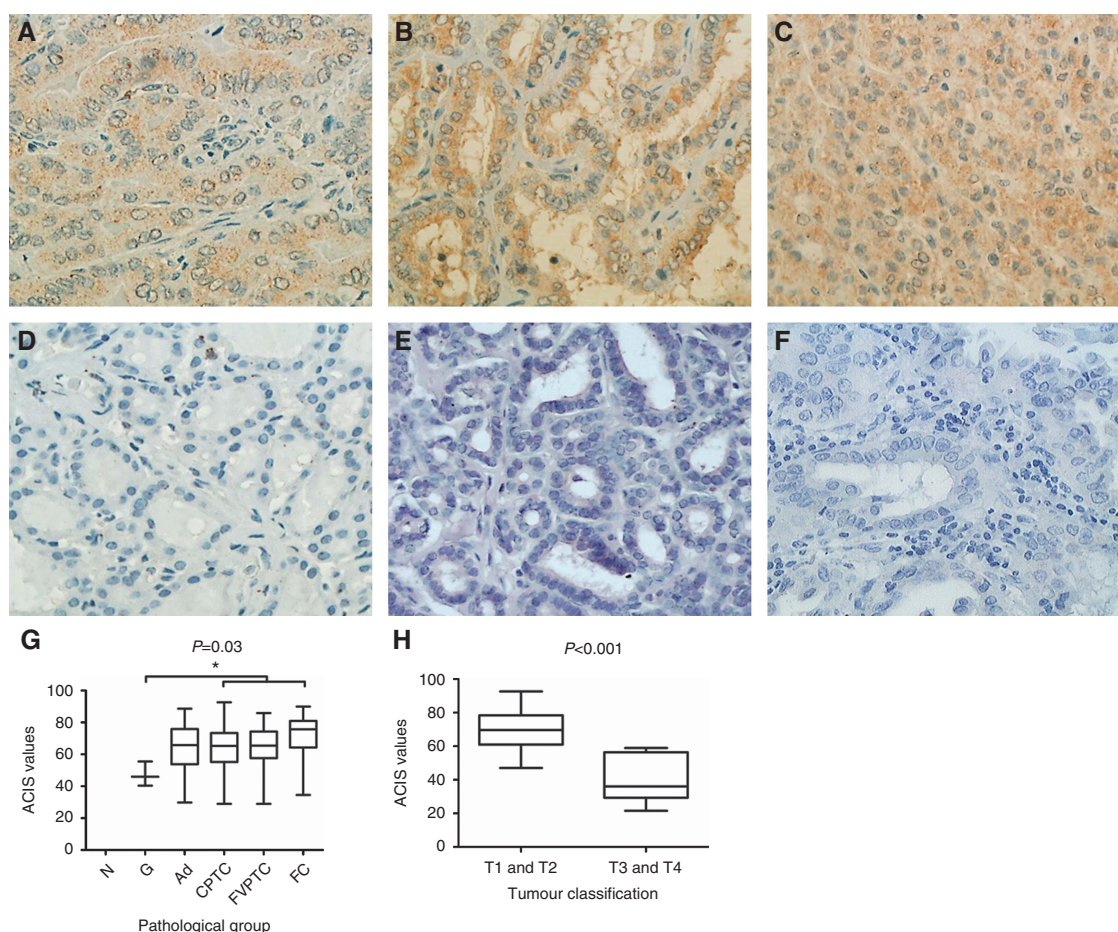


Figure 4. Immunohistochemistry with the scFv-C1 antibody in thyroid tissue. Immunostaining of the scFv-C1 antibody demonstrates reaction in thyroid tumour cells: (A) papillary thyroid carcinoma, (B) follicular variant of papillary thyroid carcinoma and (C) follicular carcinoma. Negative staining was observed in (D) normal thyroid, (E) papillary thyroid carcinoma with irrelevant scFv-C1, specific to *Mycobacterium tuberculosis* and (F) papillary carcinoma without scFv-secondary Ab alone ($\times 400$). The graphics show the average of the staining area for each pathological group (G) (normal (N), goitre (G), adenoma (Ad), papillary thyroid carcinoma (CPTC), follicular variant of papillary thyroid carcinoma (FVPTC) and follicular carcinoma (FC)), (H) ACIS immunostaining score low risk vs high risk. Significance was determined by the nonparametric, Mann-Whitney test.

Table 1. Diagnostic value of scFv-C1 discriminating different histopathological subtypes

Variable	P*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CPTC + FVPTC + FC × benign	<0.0001*	55	82	77	62
CPTC + FVPTC + FC × goitre	<0.0001*	55	94	95	95
CPTC × goitre	<0.0001*	53	94	87	72
FVPTC × goitre	<0.0001*	50	94	83	75
FC × goitre	<0.0001*	60	94	89	73
CPTC + FVPTC + FC × adenoma	0.0024*	29	45	20	57
CPTC × adenoma	0.0291*	29	47	43	33
FVPTC × adenoma	0.0624	50	71	50	71
FC × adenoma	0.0035*	29	40	38	31
CPVF × FVPTC	1000	53	50	57	45
CPVF × CF	0.6526	53	40	44	44
CPTC + FVPTC + FC					
Low risk × high risk	0.0108*	66	64	78	50

Abbreviations: CPTC = classic type PTC; FC = follicular carcinoma; FVPTC = follicular variant PTC; NPV = negative-predictive value; PPV = positive-predictive value. Fisher's exact test, *P<0.05.

Table 2. Carcinoma type, T category and score immunostaining

TNM	P/N	Score
Papillary carcinoma		
1	14/18	85.42
2	3/4	78.01
3	2/7	35.72
4	1/4	30.89
Follicular variant papillary carcinoma		
1	7/10	75.27
2	5/9	79.09
3	1/3	40.31
4	2/6	37.90
Follicular carcinoma		
1	4/7	79.75
2	2/3	83.04
3	3/5	65.24
4	1/3	51.23

Abbreviations: P = positive number of patients; Score = ACIS immunostaining media; TNM = classification of malignant tumours.

position of the corresponding spot on the 2D gel (Figure 5C), which was identified with accession number in UniProtKB/SwissProt as Q5VV17.

DISCUSSION

Successful combinatorial antibodies have been obtained against various antigens for different diseases using phage display technology (Miller *et al*, 2005; Wolf *et al*, 2006; Flego *et al*, 2007). However, this is the first investigation, to our knowledge, that resulted in a specific scFv antibody associated with well-differentiated thyroid carcinomas, mainly recognising early tumour

stages. In addition, we have constructed an immune antibody library and scFvs were selected against antigens of the entire cell surface, which differentiates from most previously described selections of combinatorial antibodies that have been carried out using known and previously isolated antigens (Rauchenberger *et al*, 2003; Martinez-Torrecuadrada *et al*, 2005; Gorbenko *et al*, 2009). A completely cell-based procedure for antibody selection is an advantage for many membrane-associated proteins, as these proteins are often difficult to express and purify. Moreover, their complex structures and native conformations are maintained (Lipes *et al*, 2008).

Our immunohistochemistry results demonstrated that the selected scFv-C1 antibody was able to distinguish follicular variants of PTCs from simple benign hyperplasia. In addition, it helped identify smaller isolated T1 and T2 lesions, which may have a better response to therapy (Varandas *et al*, 2007). The identification of low-risk tumours is essential to the management of patients with papillary thyroid cancer (Cooper *et al*, 2009).

Mass spectrometry analysis of the spot immunostained with scFv-C1 led to the identification of an OTU domain-containing protein 1, alternatively named DUBA-7, which belongs to the deubiquitinating enzyme family (DUBs). DUBs are important enzymes that remove the covalently attached ubiquitin from proteins, thereby controlling substrate activity and/or abundance (Makarova *et al*, 2000). Because of the broad involvement of the ubiquitin-proteasome proteolysis in the regulation of protein turnover, this pathway may have a vital role in cancer development and progression (Yang, 2007). Several types of deubiquitinating enzymes have been associated with development and progression of cancers (Yang, 2007) and have been found to be upregulated in prostate cancer (Priolo *et al*, 2006), follicular lymphomas (Schwickart *et al*, 2010), colon and breast carcinomas (Popov *et al*, 2007). However, to the best of our knowledge, this is the first evidence that DUBA-7 has a role in thyroid carcinogenesis.

In summary, we constructed a human scFv fragment antibody library displayed in the filamentous phage capsid, aiming to select ligands to the surface of thyroid cancer cells. We were able to identify a highly reactive antibody that distinguished benign follicular lesions from carcinomas, and that was also associated with lower-risk patients. Finally, we have demonstrated that the scFv-C1 antibody recognises a deubiquitinating enzyme, OTU domain-containing protein 1, that is also overexpressed in other

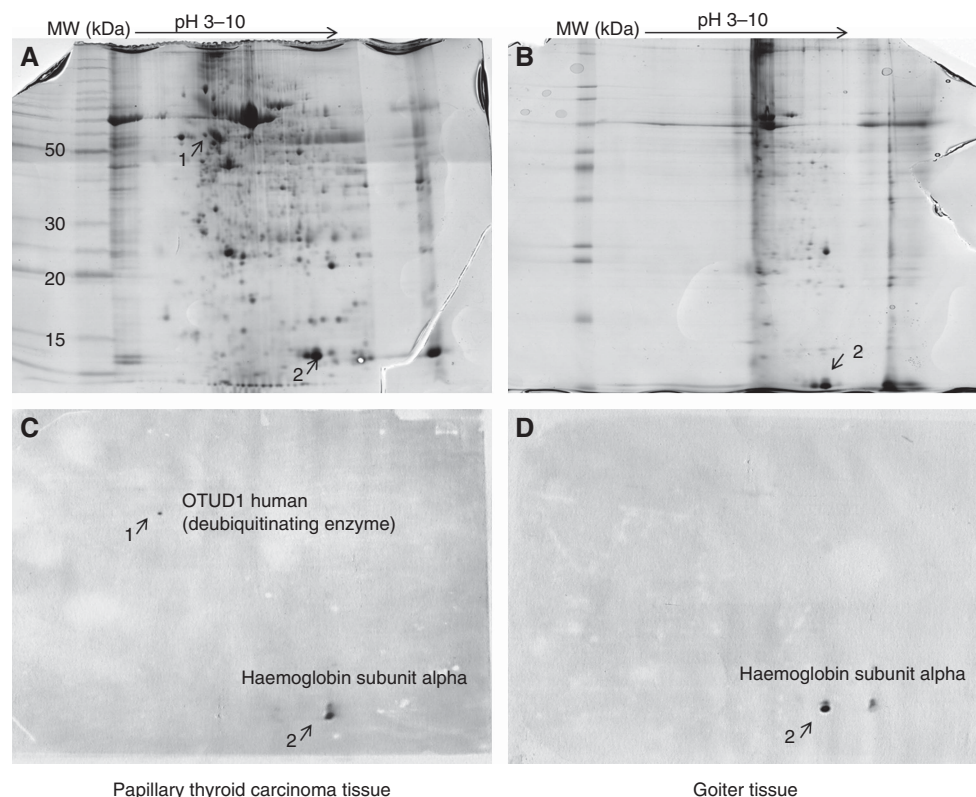


Figure 5. The two-dimensional gel electrophoresis of papillary thyroid carcinoma tissue (A) and goitre tissue (B). The sizes of the molecular weight markers (MW) are shown on the left side of panel A. The pH range was 3–10. Western blot analysis of the scFv-C1 antibody in papillary thyroid carcinoma (C) and goitre (D); the deubiquitinating enzyme—OTUD1 appeared only in carcinoma.

cancers. This specific ligand may also be a potential therapeutic agent or drug carrier for future treatment strategies of thyroid cancer.

ACKNOWLEDGEMENTS

The present work was supported by the Brazilian funding agencies: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We thank Dr Jose Vassallo (A.C. Camargo) for performing IHC analysis, and the A. C. Camargo Hospital for providing tissue microarrays.

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