Proteomic analysis of differentially expressed proteins in normal human thyroid cells transfected with PPFP

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Abstract

The fusion gene encoding the thyroid-specific transcription factor PAX8 and peroxisome proliferator-activated receptor γ (PPAR γ (PPARG)) (designated as the PPFP gene) is oncogenic and implicated in the development of follicular thyroid carcinoma (FTC). The effects of PPFP transfection on the biological characteristics of Nthy-ori 3-1 cells were studied by MTT assay, colony formation, soft-agar colony formation, and scratch wound-healing assays as well as by flow cytometry. Furthermore, the differentially expressed proteins were analyzed on 2-DE maps and identified by MALDI-TOF-MS. Validation of five identified proteins (prohibitin, galectin-1, cytokeratin 8 (CK8), CK19, and HSP27) was determined by western blot analysis. PPFP not only significantly increased the viability, proliferation, and mobility of the Nthy-ori 3-1 cells but also markedly inhibited cellular apoptosis. Twenty-eight differentially expressed proteins were identified, among which 19 proteins were upregulated and nine proteins were downregulated in Nthy-ori 3-1^{PPFP} (Nthy-ori 3-1 cells transfected with PPFP). The western blot results, which were consistent with the proteome analysis results, showed that prohibitin was downregulated, whereas galectin-1, CK8, CK19, and HSP27 were upregulated in Nthy-ori 3-1^{PPFP}. Our results suggest that PPFP plays an important role in malignant thyroid transformation. Proteomic analysis of the differentially expressed proteins in PPFP-transfected cells provides important information for further study of the carcinogenic mechanism of PPFP in FTCs.

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Introduction

Thyroid cancer is the most common malignant tumor of the endocrine organs, and its incidence has steadily increased over the past few decades (Davies & Welch 2006, Chen *et al.* 2009, Wang & Wang 2012). Follicular thyroid carcinoma (FTC), the second most common differentiated thyroid cancer, shows more aggressive clinicopathological features and a greater rate of recurrence than papillary thyroid carcinomas (PTCs). Additionally, FTCs are more prone to metastasize to the lung, bone, brain, and liver in

comparison with PTCs (Baloch & LiVolsi 2007, Witt 2008). Moreover, FTC can only be diagnosed if capsule and vascular invasion are found in the surgical resection specimens, making it difficult to diagnose by fine needle aspiration (FNA) biopsy (Baloch & LiVolsi 2007). Understanding the genetic alterations in FTCs and their mechanisms is fundamentally important in developing new molecular markers for earlier diagnosis and novel therapeutic targets.

Kroll *et al.* (2000) were the first to report the genetic rearrangement of the thyroid-specific transcription

factor PAX8 and peroxisome proliferator-activated receptor γ (PPAR γ (PPARG)), which results in the overexpression of the PAX8/PPAR γ fusion protein (PPFP) in FTC. Later studies showed that this rearrangement was mainly detected in FTC and accounted for about 29–62% of all FTC cases by immunohistochemistry or RT-PCR (Dwight *et al.* 2003, Nikiforova *et al.* 2003, Reddi *et al.* 2007, Algeciras-Schimmich *et al.* 2010). The PAX8/PPAR γ fusion gene has been demonstrated to be an oncogene that is closely related to the occurrence and development of FTC. Research concerning the oncogenic mechanism of PAX8/PPAR γ rearrangement has concentrated on its effects on the function of wild-type PPAR γ and PAX8 (Gregory Powell *et al.* 2004, Reddi *et al.* 2007). Despite some progress, the role of PPFP in the tumorigenesis of thyroid cancer remains unclear. In this study, thyroid cells were transfected with PPFP, and the changes in their biological characteristics were studied to determine the role of PPFP in the malignant transformation of thyroid cells. In order to find proteins that interact with PPFP, the proteins that were differentially expressed in PPFP-transfected cells were identified by two-dimensional electrophoresis and mass spectrometry.

Materials and methods

Construction of the recombinant vector containing the human PPFP gene

The PAX8 and PPAR γ genes were isolated from the PAX8-pOTB7 and PPAR γ -pCMV-SPORT6 plasmids (Genechem, Shanghai, China) respectively and amplified by PCR. The two genes were then ligated together and subcloned into the eukaryotic expression vector pEGFP-C1. The resulting recombinant vector pEGFP-C1-PAX8/PPAR γ was designed to express the PAX8/PPAR γ fusion gene. The PCR-amplified PPFP gene was confirmed by endoenzyme digestion and DNA sequencing (Fig. 1A).

Cell culture and transfection

To achieve stable and efficient expression, recombinant lentiviral vector was transferred into subjected cells. First, the PAX8/PPAR γ fusion gene was isolated from the EGFP-C1-PPFP plasmid and amplified by PCR. The fusion gene was then subcloned into the recombinant lentiviral vector pGC-FU (Genechem), which contains a FLAG tag, to generate the lentiviral expression vector pGC-FU-PPFP. Recombinant lentiviruses, which contained the PPFP gene and FLAG tag, were produced by 293T cells following the

co-transfection of pGC-FU-PPFP and the packaging plasmids pHelper1.0 and pHelper2.0 (Genechem). The viral titer was measured after collecting and concentrating the viral supernatant. PCR was used to screen for PPFP-positive colonies (Fig. 1B). In order to verify the expression of the PPFP gene, antibodies against FLAG and PPAR were used to detect PPFP expression in the 293T cells by western blot analysis (Fig. 1C and D). The targeted cells, Nthy-ori 3-1 (HPACC, Salisbury, UK), originate from normal human thyroid cells that have been immortalized by the expression of SV40 large T antigen. Nthy-ori 3-1 cells were transfected by pGC-FU-PPFP to establish a stable Nthy-ori 3-1^{PPFP} cell line. To evaluate the efficacy of transfection, we examined the expression of green fluorescent protein (GFP) and found that the lentiviral supernatants could effectively and stably infect Nthy-ori 3-1 cells, as shown in Fig. 2. Meanwhile, Nthy-ori 3-1^{vector} cells, which were transfected with empty lentiviral vector and untransfected Nthy-ori 3-1 cells, were included as control cell lines. Cells were grown at 37 °C in a humidified 5% CO₂ incubator with RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). The medium was changed every 2–3 days, and the cells were passaged every 3–5 days.

MTT, colony formation, soft-agar colony formation, scratch wound-healing assays, and flow cytometry

To study the changes in the biological characteristics of the Nthy-ori 3-1 cells after PPFP gene transfection, we analyzed cell proliferation with the MTT assay, colony number with the plate colony forming assay, colony-forming efficiency with the soft-agar colony formation assay, migration and movement capacity with the scratch wound-healing assay, and apoptosis and cell cycle with flow cytometry. For each of the three groups (Nthy-ori 3-1^{PPFP}, Nthy-ori 3-1^{vector}, and Nthy-ori 3-1), cells in the logarithmic phase were used in all experiments and incubated at 37 °C in 5% CO₂. For the MTT assay, cells were incubated with 0.5 mg/ml MTT for 4 h. The formazan crystals produced by the living cells in the culture were dissolved with 100 μ l dimethyl sulfoxide, and the absorbance (OD value) was measured at 570 nm using a 96-well plate reader at different time points (0, 24, 48, 72, and 96 h). For the plate colony-forming assay, cells were seeded onto six-well plates at 300 cells/well and incubated with complete medium at 37 °C in 5% CO₂ for 14 days. The number of surviving colonies (a colony was defined to contain >50 cells) was counted, and the difference in colony formation between the three

Figure 1 Construction and production of the recombinant vector containing the human PPFP gene. (A) Lane 1: marker, lane 2: pEGFP-C1 vector, lane 3: pEGFP-C1 digested with XhoI and KpnI showed a band of about 4.7 kb, which is consistent with the size of the desired fragment. (B) PCR assay to identify PPFP-positive colonies (lane 1: negative control, lane 2: positive control, and lane 3: marker). Lanes 4, 6, and 9 (PPFP-positive colonies) had a 565 bp PCR fragment, which was designed to detect the presence of the PPFP gene. (C and D) Western blot detection of PPFP expression in 293T cells using an anti-mouse FLAG antibody and an anti-mouse PPAR antibody respectively. Lane 1: untransfected 293 T cells, lane 2: 293T cells transfected with empty vector, and lanes 3 and 4: 293T cells transfected with PPFP showed a band with a molecular weight of about 90 kDa, which corresponds to the size of PPFP.

experimental groups was determined. For the soft-agar colony formation assay, a two-layer soft-agar culture was prepared with a bottom layer of 0.6% agarose and a top layer of 0.35% agarose. Cells were seeded onto six-well plates at a density of 2×10^4 cells/well and

incubated at 37 °C in 5% CO₂ for 2 weeks. The number of colonies (diameter > 75 μm or cell count > 50) was counted for further analysis. The rate of colony formation was calculated by dividing the number of colonies with the total cell count per well. For the

Figure 2 PPFP expression in Nthy-ori 3-1 cells transfected with PPFP by green fluorescent protein detection: Abundant green fluorescence was observed by fluorescence microscopy, and more than 90% of Nthy-ori 3-1 cells stabilized and highly expressed the targeted gene after 12 days. A, B, and C represent untransfected cells, Nthy-ori 3-1^{vector}, and Nthy-ori 3-1^{PPFP} respectively. Row 1: fields viewed under the light microscope; row 2: fields viewed under the fluorescent microscope.

scratch wound-healing assay, cells were seeded at a density of 5×10^5 cells/well in six-well plates, which were precoated overnight at 4 °C with PBS containing 10 µg/ml fibronectin. Then, the cells were incubated in 5% CO₂ at 37 °C until a confluent monolayer formed. A scratch of about 300–500 µm was made manually by scraping the cell monolayer with a 200-µl pipette tip. After washing twice with PBS, the cells were cultured in serum-free medium. Images were captured at different time points (0, 12, 24, 36, and 48 h), and the distance that the cells migrated to the wounded region was measured. For flow cytometry, the prepared cells were collected and digested into single-cell suspensions using 0.25% trypsin. Then, the cells were centrifuged at 500 g for 5 min and washed with PBS (0.01 M, pH 7.4) twice. Seventy percent ethanol stored at 4 °C was used to fix the cells for 24 h before they were fully shaken and dispensed. The plates were incubated with 0.5% Triton X-100 (Sigma) and 0.05% RNase (Sigma) in 1 ml PBS at 37 °C for 30 min and then centrifuged at 1259 g for 5 min. The cells were stained with 50 µg/ml propidium iodide (Sigma) at room temperature for 30 min, and the cell number was adjusted to 1×10^6 /ml. Samples were immediately analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

IPG-2D PAGE

The three cell lines Nthy-ori 3-1^{PFP}, Nthy-ori 3-1^{vector}, and Nthy-ori 3-1 were lysed in 100 µl lysis buffer (7 M urea, 2 M thiourea, 100 mM dithiothreitol (DTT), 4% CHAPS, 40 mM Tris, 2% pharmalyte, and 1 mg/ml DNaseI). The lysates were centrifuged at 12 000 g for 30 min at 4 °C, and the supernatant was collected and stored at –80 °C until use. The total protein concentration was determined using a 2D Quantification kit (Amersham Biosciences) before two-dimensional electrophoresis. Triplicate gels were prepared for each cell line, and a total amount of 1000 µg protein per gel was analyzed. Protein samples were diluted to 450 µl with rehydration solution (8 M urea, 4% CHAPS, 40 mM Tris, 40 mM DTT, 0.5% IPG buffer, and trace bromophenol) and applied to IPG strips (pH 3–10L, 240×3×0.5 mm, Amersham Biosciences), which were rehydrated for 12 h at 30 V. The proteins were then focused successively for 1 h at 500 V, 1 h at 1000 V, and 8.5 h at 8000 V for a total of 69 920 Vh on an IPGphor system (Amersham Biosciences). Focused IPG strips were equilibrated for 15 min in 10 ml solution A (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 1% SDS, and 0.2% DTT) and then for an additional 15 min in 10 ml solution B (the same as

solution A except that DTT was replaced with 3% iodoacetamide). Protein separation on the second dimension was achieved by SDS–PAGE using 12.5% homogenous gels and the Ettan Dalt II system (Amersham Biosciences). After SDS–PAGE, the protein spots were visualized via Blue Silver stain, a modification of Neuhoff's colloidal Coomassie Blue G-250 stain (Amersham Biosciences), according to Candiano *et al.* (2004).

Image analysis

The stained 2-DE gels were scanned with LabScan software using ImageScanner (Amersham Biosciences) with the resolution set at 300 dpi. The spot-intensity calibration, spot detection, background subtraction, matching, 1-D calibration, and the establishment of average gel were accomplished with the PDQuest system (Bio-Rad Laboratories). The amount of stained protein within the individual spots was quantified by calculating the spot volume after normalizing the image using the total spot volume normalization method and multiplying by the total area of all spots. The calculation of the theoretical molecular weight and *pI* value of the identified protein spots was based on algorithms included in the PDQuest analysis software package (Bio-Rad Laboratories). Proteins were classified as being differentially expressed when the spot intensities showed a ≥ 2 -fold difference between the three cell lines. Significant differences in protein expression levels were determined using the Student's *t*-test with $P < 0.05$.

Protein identification by MALDI-TOF-MS and database analysis

The differentially expressed protein spots were excised from the stained gels using a punch, and in-gel trypsin digestion was performed as described (Chen *et al.* 2008). After destaining with 100 mM sodium thiosulfate and 30 mM potassium ferricyanide, the protein-containing gel spots were subsequently reduced, alkylated, and dried. Then, the dried gel pieces were incubated in the digestion solution, which consisted of 40 mM ammonium bicarbonate, 9% can, and 20 µg/ml trypsin (Sigma) for 12 h at 37 °C. The purified tryptic peptide mixture was mixed with an α -cyano-4-hydroxycinnamic acid matrix solution and vortexed lightly. Then, 1 µl mixture was analyzed with a Voyager System DE-STR 4307 MALDI-TOF Mass Spectrometer (ABI, Carlsbad, CA, USA) to obtain a peptide mass fingerprint (PMF). To search the PMF map database, Mascot Distiller was used to obtain the monoisotopic peak list from the raw mass spectrometry

files. Proteins were identified by searching the Swiss-Prot database via the Mascot search engine (<http://www.matrixscience.com/>). The search parameters were set as following: the mass tolerance was ± 0.5 Da; the number of missed cleavage sites allowed was up to 1; the cysteine residue was modified as carbamidomethyl-cys; variable modifications included oxidation (M); the minimum number of matched peptides was 5; the species was selected as HOMO SAPIENS (HUMAN); the peptide ion was $[M+H]^+$; mass values was set as monoisotopic; the search range was within the experimental pI value ± 0.5 pH unit and experimental $Mr \pm 20\%$; isotope masses were used; and protein scores provided by the Mascot program that were >56 were considered significant ($P < 0.05$).

Western blot analysis

Cells were collected from the three groups: Nthy-ori 3-1^{PPFP}, Nthy-ori 3-1^{vector} and Nthy-ori 3-1. Total protein samples were prepared as mentioned earlier, and the concentration was determined using the BCA Protein Assay Kit (Amersham Biosciences). Western blot analysis was performed as described previously (Yang *et al.* 2006). Briefly, 40 μ g lysate was separated by 10% SDS–PAGE and transferred to a PVDF membrane. Blots were blocked with 5% nonfat dry milk for 2 h at room temperature and then incubated with a rabbit polyclonal anti-human prohibitin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal anti-human galectin-1 antibody (Santa Cruz), a mouse monoclonal anti-human cytokeratin 8 (CK8) antibody (Santa Cruz), a mouse monoclonal anti-human CK19 antibody (Santa Cruz), or a rabbit monoclonal anti-human HSP27 antibody (Santa Cruz) overnight at room temperature. HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (Santa Cruz) was incubated with the blots in sequence at room temperature for 2 h. The signal was visualized with ECL detection reagent and quantified by densitometry using the ImageQuant image analysis system (Storm Optical Scanner, Molecular Dynamics, Sunnyvale, CA, USA). β -Actin was detected simultaneously using a mouse anti- β -actin antibody (Sigma) as a control.

Statistical analysis

The statistical analyses were performed using SPSS 13.0 software. Single comparisons were performed using the Student's *t*-test or Mann–Whitney's *U* test, and multiple comparisons were performed using one-way ANOVA. *P* values < 0.05 were considered statistically significant.

Results

The effect of PPFP on the biological characteristics of Nthy-ori 3-1 cells

In the MTT assay, the absorbance values of Nthy-ori 3-1^{PPFP} at 48, 72, and 96 h were significantly higher than those of Nthy-ori 3-1^{vector} and Nthy-ori 3-1 ($P < 0.01$, Fig. 3A). Compared with the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells, the Nthy-ori 3-1^{PPFP} cells formed more colonies after 14 days of culture ($P < 0.01$). However, there was no difference between the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells ($P > 0.05$, Fig. 3B). The soft-agar colony formation assay, which was used to examine anchorage-independent growth, showed that all three cell lines were capable of colony formation. However, the colony formation rates for Nthy-ori 3-1^{vector} and Nthy-ori 3-1 were significantly less than that for Nthy-ori 3-1^{PPFP} ($P < 0.01$, Fig. 3C). The migration of Nthy-ori 3-1 cells transfected with pGC-FU-PPFP was greater than that of the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells. The results showed a significant increase in cell migration for the Nthy-ori 3-1^{PPFP} cells after 24 h, and the scratch was almost healed after 48 h, while scratches were only partially healed for the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells, as shown in Fig. 3D. Flow cytometric analyses demonstrated that the apoptotic percentage of the Nthy-ori 3-1^{PPFP} cells was significantly less than that of the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells ($P < 0.01$, Fig. 4). The number of Nthy-ori 3-1^{PPFP} cells in G₀/G₁ phase decreased significantly ($P < 0.01$) but that in S and G₂/M phase increased significantly ($P < 0.01$) in comparison with both the Nthy-ori 3-1^{vector} and the Nthy-ori 3-1 cells. However, there was no significant difference between the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells.

Detection of differentially expressed proteins in Nthy-ori 3-1 cells transfected with PPFP by 2-DE

Comparative proteomics analysis was performed to identify the differentially expressed proteins in the Nthy-ori 3-1^{PPFP}, Nthy-ori 3-1^{vector}, and Nthy-ori 3-1 cells. 2-DE of each cell line was performed in triplicate to obtain a reliable map with a clear background, high definition, and repeatability. The protein spots detected in the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells were not significantly different after comparing their average 2-DE maps. Compared with the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells, 38 protein spots detected in the Nthy-ori 3-1^{PPFP} cells were consistently different (≥ 2 -fold) in triplicate experiments. As shown in

Figure 3 Physiological impact of PPFP on thyroid cells. (A) MTT assay. Nthy-ori 3-1^{PPFP} cells had higher absorbance values at 48, 72, and 96 h compared with Nthy-ori 3-1^{vector} and untransfected Nthy-ori 3-1 cells (* $P < 0.01$). (B) Soft-agar colony formation assay. Nthy-ori 3-1^{PPFP} cells formed more colonies compared with Nthy-ori 3-1^{vector} and untransfected Nthy-ori 3-1 cells (* $P < 0.01$). (C) Plate formation assay (1, Nthy-ori 3-1; 2, Nthy-ori 3-1^{vector}; and 3, Nthy-ori 3-1^{PPFP}). Nthy-ori 3-1^{PPFP} cells had a higher colony formation rate compared with Nthy-ori 3-1^{vector} and untransfected Nthy-ori 3-1 cells (* $P < 0.01$). (D) Scratch wound-healing assay (1, Nthy-ori 3-1; 2, Nthy-ori 3-1^{vector}; 3, Nthy-ori 3-1^{PPFP}). The scratch was almost healed in Nthy-ori 3-1^{PPFP} cells after 48 h, while scratches in Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells were only half-healed.

Fig. 5, increased expression of 19 protein spots and decreased expression of nine protein spots were found in the 28 differentially expressed protein spots detected in the Nthy-ori 3-1^{PPFP} cells, which were subsequently identified by MS.

Identification of the differentially expressed proteins by MS

All the differentially expressed protein spots were excised from stained gels, *in situ* digested with trypsin, and analyzed by MALDI-TOF-MS. From a total of 28 differentially expressed protein spots identified by MS, the levels of 19 proteins increased and those of nine proteins decreased in Nthy-ori 3-1^{PPFP} cells respectively as shown in Table 1. The MALDI-TOF mass spectrometry map and database query result of a representative spot (protein spot 6) are shown in Fig. 6. Data for 15 monoisotopic peaks were input into the Mascot program to search the Swiss-Prot database, and the query result showed that protein spot 6 was prohibitin.

Validation of the identified proteins

To confirm the identification of the differentially expressed proteins identified using the 2-DE maps, the expression of five identified proteins (prohibitin,

galectin-1, CK8, CK19, and HSP27) in Nthy-ori 3-1, Nthy-ori 3-1^{vector}, and Nthy-ori 3-1^{PPFP} cells was detected by western blot. As shown in Fig. 7, the expression of galectin-1, CK8, CK19, and HSP27 was significantly upregulated in Nthy-ori 3-1^{PPFP} cells compared with Nthy-ori 3-1^{vector} and Nthy-ori 3-1

Figure 4 Effect of PPFP on the cell cycle and apoptosis of Nthy-ori 3-1 cells. The number of Nthy-ori 3-1^{PPFP} cells in G₀/G₁ phase decreased significantly but the number in S and G₂/M phase increased significantly compared with Nthy-ori 3-1 and Nthy-ori 3-1^{vector} cells ($P < 0.01$). The apoptotic percentage of Nthy-ori 3-1^{PPFP} cells was significantly less than Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells (* $P < 0.01$).

Figure 5 (A) Representative 2-DE maps of Nthy-ori 3-1^{PPFP}, Nthy-ori 3-1^{vector}, and Nthy-ori 3-1 cells. The 28 differentially expressed protein spots that were identified by MS are marked with arrows. (B) Close-up image of differentially expressed protein spots in the three groups of cells.

cells ($P < 0.05$), while that of prohibitin decreased significantly in Nthy-ori 3-1^{PPFP} cells ($P < 0.05$). Also, no obvious difference was observed between the Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells, which is consistent with the results from the proteomic analysis.

Discussion

Follicular cancers, which comprise 10–15% of total thyroid cancers, are more difficult to detect at early stages even by FNA. Recently, the detection of somatic mutations has been proposed for the preoperative FNA diagnosis of differentiated thyroid cancer (Cerutti 2011). As seen in most malignant neoplasms, thyroid cancers usually are associated with specific genetic abnormalities; two major genetic alterations (RAS mutation and PAX8/PPAR γ rearrangement) are found in FTC. Interestingly, these two genetic backgrounds rarely overlap in the same tumor, suggesting that two independent pathways are involved in the development of FTC (Nikiforova *et al.* 2003). While the Ras mutation is not restricted to a particular histological type, PAX8/PPAR γ rearrangement mainly presents

with follicular neoplasms and a small portion of follicular variants of papillary thyroid cancers (Dwight *et al.* 2003, Nikiforova *et al.* 2003, Kroll 2004, Lacroix *et al.* 2004, 2005, Koenig 2010, Nikiforov & Nikiforova 2011). Because the expression of PPFP indicates a favorable clinical presentation and prognosis, FTCs presenting with PAX8/PPAR γ rearrangement are thought to be a less aggressive subtype (Sahin *et al.* 2005). Although it is expressed at a lower frequency in follicular thyroid adenoma, PPFP is believed to play an important role in thyroid tumorigenesis, probably at an early stage (Nikiforova *et al.* 2002, Gregory Powell *et al.* 2004, Reddi *et al.* 2007). However, the role of PPFP in thyroid cancer is not fully understood.

Based on the successful construction of recombinant lentiviral expression vector containing PPFP (pGC-FU-PPFP), we studied the biological characteristics of SV40 large-T-antigen-immortalized normal human-differentiated thyroid cells (Nthy-ori 3-1) after transfection with PPFP. Our results showed that the expression of PPFP in Nthy-ori 3-1 cells could prompt cellular proliferation, inhibit apoptosis, induce cell

Table 1 Differential proteins in Nthy-ori 3-1^{PPFP} cells identified by MALDI-TOF MS

Spot no.	Swiss-Prot AC	Protein name	MW (Da)	pI	Score ^a	Coverage (%)	Expression in Nthy-ori 3-1 ^{PPFP}	Functional classification
1	P06576	ATP synthase subunit β , mitochondrial	56 525	5.26	155	54	↑	ATP synthesis
2	P07237	Protein disulfide isomerase	57 480	4.76	138	25	↑	Metabolism
3	P30101	Protein disulfide isomerase A3	57 146	5.98	227	48	↑	Protein folding
4	P20338	Ras-related protein RAB4A	24 141	5.82	74	33	↓	Protein transport
5	Q9H239	Matrix metalloproteinase-28	59 187	9.70	92	38	↑	Tissue homeostasis and repair
6	P35232	Prohibitin	29 843	5.57	262	64	↓	DNA replication, regulating proliferation
7	Q99714	3-Hydroxyacyl-CoA dehydrogenase type-2	27 134	7.66	167	54	↓	tRNA processing
8	P68133	Actin, α skeletal muscle	42 366	5.23	78	30	↓	Cytoskeleton
9	P51530	DNA2-like helicase	122 048	7.95	61	20	↑	DNA replication
10	Q8N8E3	Coiled-coil domain-containing protein 46	113 192	6.25	67	26	↑	–
11	P05787	Keratin, type II cytoskeletal 8	53 671	5.52	200	43	↑	Cytoskeleton
12	P67936	Tropomyosin α -4 chain	28 619	4.67	59	36	↓	Cytoskeleton
13	Q14457	Beclin-1	52 377	4.83	111	36	↓	Cell cycle
14	P09382	Galectin-1	15 048	5.34	118	61	↑	Apoptosis, proliferation, cell differentiation
15	P09211	Glutathione S-transferase P	23 569	5.43	127	48	↑	Anti-apoptosis
16	P06733	α -Enolase	47 481	7.01	174	46	↑	Transcriptional repressor
17	P30084	Enoyl-CoA hydratase, mitochondrial	31 823	8.34	113	29	↑	Metabolism
18	P08727	Keratin, type I cytoskeletal 19	44 065	5.04	245	43	↑	Cytoskeleton
19	P21964	Catechol O-methyltransferase	30 474	5.26	87	39	↓	Metabolism
20	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	37 464	8.97	93	38	↓	mRNA processing
21	Q9Y241	HIG1 domain family member 1A	10 136	9.79	98	68	↑	Stress response
22	Q562R1	β -Actin-like protein 2	42 318	5.29	65	21	↑	Cytoskeleton
23	P62937	Peptidyl-prolyl <i>cis-trans</i> isomerase A	18 229	7.68	146	69	↑	Protein folding
24	P04792	Heat-shock protein 27	22 826	5.98	159	61	↑	Stress resistance and actin organization
25	P23528	Cofilin-1	18 719	8.22	91	56	↓	Cell morphology and cytoskeletal organization
26	P04075	Fructose-bisphosphate aldolase	39 851	8.30	152	55	↑	Glycolysis and gluconeogenesis
27	P46940	Ras GTPase-activating-like protein IQGAP1	189 761	6.08	118	35	↑	Signal transduction
28	P30040	Endoplasmic reticulum protein ERp29	29 032	6.77	78	31	↑	Protein secretion

^aMascot score is 56. Protein scores by Mascot searching > 56 were considered significant ($P < 0.05$).

cycle transition, and spur cellular migration. All these data indicate that PPFP can provide a significant growth advantage and contribute to malignant transformation in thyroid cancers. Consistent with other studies, our results also demonstrated that PPFP plays an important role in stimulating the malignant cellular proliferation, invasion, and metastasis of thyroid cancer (Gregory Powell *et al.* 2004, Au *et al.* 2006, Espadinha *et al.* 2007). Because all these studies are based on cell lines that were already immortalized, such as Nthy-ori 3-1, whether PPFP alone is sufficient

to trigger tumorigenesis or whether other molecular events are involved remains unclear. It is essential to develop *in vivo* animal models with PPFP expression in the thyroid to demonstrate the role of PPFP in tumorigenesis. As shown in our table, 28 differentially expressed proteins, including 19 upregulated and nine downregulated proteins, were identified by a proteomic approach in Nthy-ori 3-1^{PPFP} cells. These differentially expressed proteins indicated that the involvement of PPFP in FTC tumorigenesis is a complicated process that is related to proliferation, apoptosis,

Figure 6 MALDI-TOF-MS analysis of differential protein spot 6. (A) The mass spectrum of protein spot 6 identified this protein as prohibitin according to the matched peaks. (B) Possible matched proteins to the spectral masses are presented as multiple bars with differential probability-based MOWSE scores.

differentiation, metabolism, cell cycle regulation, the cytoskeleton, and signal transduction. Moreover, five differentially expressed proteins (prohibitin, galectin-1, CK8, CK19, and HSP27), which were considered to be closely connected with PFP, were selected to be further analyzed by western blot to validate our 2-DE results. The western results suggest that the proteins identified by the proteomic approach are actually differentially expressed proteins involved with PFP. These findings lay a foundation for further clarification of the molecular mechanism of the occurrence and development of FTC.

Prohibitin, which is located on chromosome 17q21, is a potential tumor suppressor that is linked to early-onset breast cancer in humans. Prohibitin inhibits cell proliferation by binding with RB and consequently modulating the transcriptional activity of E2F (Wang *et al.* 1999a,b). Prohibitin can also lead to G₁/S arrest, which also favorably influences anti-proliferation.

Several studies have revealed the reduced expression of prohibitin in the early stages of prostate, gastric, and liver cancer (Seow *et al.* 2000, Gamble *et al.* 2004, Jang *et al.* 2004). However, conflicting results have reported the overexpression of prohibitin in many other cancers, such as uterine, cervix, bladder, and lung cancer (Tsai *et al.* 2006, Wu *et al.* 2007, Guo *et al.* 2012) and even in the same type of cancers (Wang *et al.* 2004, Ummanni *et al.* 2008). Franzoni *et al.* (2009) demonstrated the high expression of prohibitin in PTCs bearing the BRAF^{V600E} mutation but low expression in follicular adenoma. In our experiments, the expression of prohibitin in thyroid cells expressing PFP decreased significantly compared with untransfected cells or those transfected with empty vector. These results suggest that PFP probably accelerates cell proliferation by downregulating the expression of prohibitin in these cells. The relief of cell cycle arrest in G₀/G₁ phase and the increase in the number of cells

Figure 7 (A) Representative results of western blot analysis for prohibitin, galectin-1, cytokeratin 8, cytokeratin 19, and HSP27 in Nthy-ori 3-1, Nthy-ori 3-1^{vector}, and Nthy-ori 3-1^{PPFP} cells. (B) Histogram of the relative changes in the expression levels of these proteins. The expression of galectin-1, cytokeratin 8, cytokeratin 19, and HSP27 was significantly upregulated in Nthy-ori 3-1^{PPFP} cells in comparison with Nthy-ori 3-1^{vector} and Nthy-ori 3-1 cells, whereas that of prohibitin decreased significantly in Nthy-ori 3-1^{PPFP} cells ($P < 0.05$).

in S phase may also attribute to the decrease in prohibitin expression due to PPFP transfection.

Galectin-1 is a developmentally regulated gene that encodes a β -galactoside-binding protein whose overexpression is associated with malignant transformation and loss of differentiation (Salvatore *et al.* 1995). Further study showed that DNA methylation is involved in the regulation of galectin-1 gene expression in rat thyroid cells (Benvenuto *et al.* 1996). The function of galectin-1 also involves developmental processes regarding fetal stromal thyroid tissue organization and thyroid epithelium maturation (Savin *et al.* 2003). High levels of galectin-1 in malignant thyroid tumors have been reported in

several studies by immunohistochemical and proteomic analysis and serum detection, suggesting its vital role in discriminating between benign and malignant thyroid lesions (Chiariotti *et al.* 1995, Xu *et al.* 1995, Torres-Cabala *et al.* 2006, Saussez *et al.* 2008). The detection of galectins in blood and tissue using FNA and large needle aspiration biopsy has been proposed for preoperative diagnosis (Carpi *et al.* 2010). Our experimental results showed that Nthy-ori 3-1 cells transfected with PPFP had increased expression of galectin-1, suggesting its potent role in the malignant transformation of thyroid cells. However, there are no studies to date that describe the mechanism by which PPFP affects the expression of galectin-1.

CKs are the major structural proteins of epithelial cells, and recent data has shown that they play an important role in cell signaling, the stress response, and apoptosis. Overexpression of some CKs, including CK8, CK18, and CK19, has been reported in several cancers and is believed to be involved in tumor progression based on several studies (Letocha *et al.* 1993, Silen *et al.* 1994, Fujita *et al.* 1999, Fukunaga *et al.* 2002, Cimpean *et al.* 2008, Liu *et al.* 2008, Kabukcuoglu *et al.* 2010). Another study reported the high expression of pan-CK, CK8, and CK19 in malignant thyroid tumors (Schroder *et al.* 1996). To date, it is not fully understood why an increase in CK8 is significantly linked to poor clinical characteristics and prognosis. In contrast to CK8, the expression of CK19 and CK19 fragments in the plasma have already been well investigated in thyroid tumors and a variety of other tumors (Flanagan *et al.* 2008, Krzeslak *et al.* 2008, Suzuki *et al.* 2010, Yang *et al.* 2010, Chung *et al.* 2011, Stewart *et al.* 2011). Further studies showed that the expression level of CK19 or CK19 fragments differ in various subtypes of thyroid tumors (Raphael *et al.* 1994, Miettinen *et al.* 1997). Most studies demonstrated that overexpression of CK19 is mainly related to papillary thyroid cancer with a positive rate, accounting for 70–100% of all cancers (Flanagan *et al.* 2008, Krzeslak *et al.* 2008, Barut *et al.* 2010, Gong *et al.* 2012). Furthermore, CK19 has been used in combination with PAX8 and HBME1 to increase the specificity of diagnosis for thyroid tumors by FNA (Schmitt *et al.* 2010, Cochand-Priollet *et al.* 2011). The upregulated expression of CK8 and CK19 was detected in Nthy-ori 3-1 cells transfected with PPFP, which may indicate that involvement of CK8 and CK19 is induced by PPFP in the malignant transformation and enhanced migration of thyroid cells.

HSP27 is a member of the small HSP family and acts as a powerful antagonist of programmed cell death in the regulation of epithelial cell growth and

differentiation (Sherman & Goldberg 2001, Sherman *et al.* 2007). Studies have shown that phosphorylated HSP27 enhances cell migration in several cell lines and tumor tissues (Morino *et al.* 1997, Piotrowicz *et al.* 1998, Rust *et al.* 1999, Shin *et al.* 2005). Reduced expression of HSP27 was reported in PTC, FTC, and multinodular goiters but enhanced expression was demonstrated in diffuse hyperplasia and follicular adenoma by proteomic analyses (Srisomsap *et al.* 2002, Bian & Tang 2010). The overexpression of HSP27 was observed in PPFP-transformed Nthy-ori 3-1 cells, which suggests that PPFP may involve in the increased cell migration of thyroid cells by HSP27.

Conclusion

In this study, our analysis of Nthy-ori 3-1 cells transfected with PPFP showed that PPFP not only increased cellular proliferation and mobility but also had an anti-apoptotic effect on Nthy-ori 3-1 cells. These results demonstrated that PPFP might play an essential role in the malignant transformation of thyroid follicular cells. A number of differentially expressed proteins were identified using a proteomic approach after thyroid follicular cells were transfected with PPFP, suggesting the involvement of multiple molecular events and signaling pathways in the process of FTC tumorigenesis. These findings not only boost the understanding of the development and progression of PTC but also generate hope for both the early diagnosis and the targeted therapy for FTC. However, further research is required to determine how these differentially expressed proteins act with PPFP with respect to its role in FTC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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