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Comparative serum proteomic analysis identified afamin as a downregulated protein in papillary thyroid carcinoma patients with non-^{131}I-avid lung metastases

Hong-Jun Song*, Yan-Li Xue*, Zhong-Ling Qiu and Quan-Yong Luo

Background The loss of ^{131}I uptake ability in metastases from differentiated thyroid carcinoma (DTC) is becoming a major obstacle in radioiodine treatment. However, there is no effective way to screen for ^{131}I uptake ability in metastases. The identification of differentially expressed proteins by serum proteomics may contribute to our understanding of the mechanisms underlying the dedifferentiation of DTC.

Materials and methods Serum samples were obtained from papillary thyroid carcinoma patients with non-^{131}I-avid lung metastases and ^{131}I-avid lung metastases. Differential protein analysis was performed using two-dimensional gel electrophoresis. Candidate protein spots showing differences in expression between the two groups were identified by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and were validated by western blotting.

Results We found that afamin is downregulated in the serum of papillary thyroid carcinoma patients with non-^{131}I-avid lung metastases.

Conclusion Afamin may be a potential serum biomarker for early screening of ^{131}I uptake ability in DTC metastases and could therefore be of value in guiding radioiodine treatment decisions. *Nucl Med Commun* 34:1196–1203 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: biomarker, lung metastases, papillary thyroid carcinoma, proteomics, radioactive iodine treatment

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Introduction

Differentiated thyroid carcinoma (DTC) is one of the most common malignancies of the endocrine system, and papillary thyroid carcinomas (PTCs) are the most frequent type of differentiated thyroid cancers [1]. Lung metastasis, which is the most common site of distant metastasis of DTC, is associated with a relatively poor prognosis [2]. Radioactive iodine (^{131}I) is a major weapon in the fight against DTC metastatic lesions. However, the uptake of ^{131}I in metastatic lesions is a prerequisite for achieving favorable therapeutic effects. Unfortunately, in clinical practice, ~30% of DTC patients with recurrent disease or metastases show dedifferentiation of malignant cells [3]. Radioiodine therapy is recommended only for ^{131}I-avid lung metastases; however, there is no obvious benefit for those with non-^{131}I-avid tumors [2]. The ability of DTC to concentrate radioactive iodine may be lost in metastatic disease, most likely because of transformation to less differentiated types (dedifferentiation) [4]. This creates a major obstacle in radioiodine treatment for which there is currently no solution. Moreover, the mechanisms underlying the dedifferentiation of DTC are not well understood.

Although a post-therapeutic ^{131}I whole-body scan (^{131}I-WBS) is an effective clinical method for assessing ^{131}I uptake ability in DTC metastases, it is usually applicable after 3–4 weeks of thyroxin withdrawal or use of rhTSH. However, patients with non-^{131}I-avid lung metastases subjected to unnecessary ^{131}I treatment should avoid the risk of high serum TSH stimulation after thyroxin withdrawal. Thus, to guide ^{131}I therapy, improve long-term survival, and reduce side effects, it is critical to screen for the ^{131}I uptake ability of lung metastases before ^{131}I therapy.

Proteomics is currently considered the most powerful screening tool for the identification of differential protein expression patterns because it enables the concomitant detection of thousands of proteins [5]. In our previous proteomics study, we compared serum protein fingerprints between PTC patients with ^{131}I-avid lung metastases and those with non-^{131}I-avid lung metastases using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and detected significantly different protein expression patterns between the two groups [6]. However, SELDI-TOF-MS does not enable the identification of differentially expressed protein peaks. Therefore, in this study, we adopted proteomic approaches, including 2-DE, for protein separation and MALDI-TOF/TOF MS for protein identification to detect and identify differentially expressed serum

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proteins between PTC patients with non-^{131}I-avid lung metastases and those with ^{131}I-avid lung metastases.

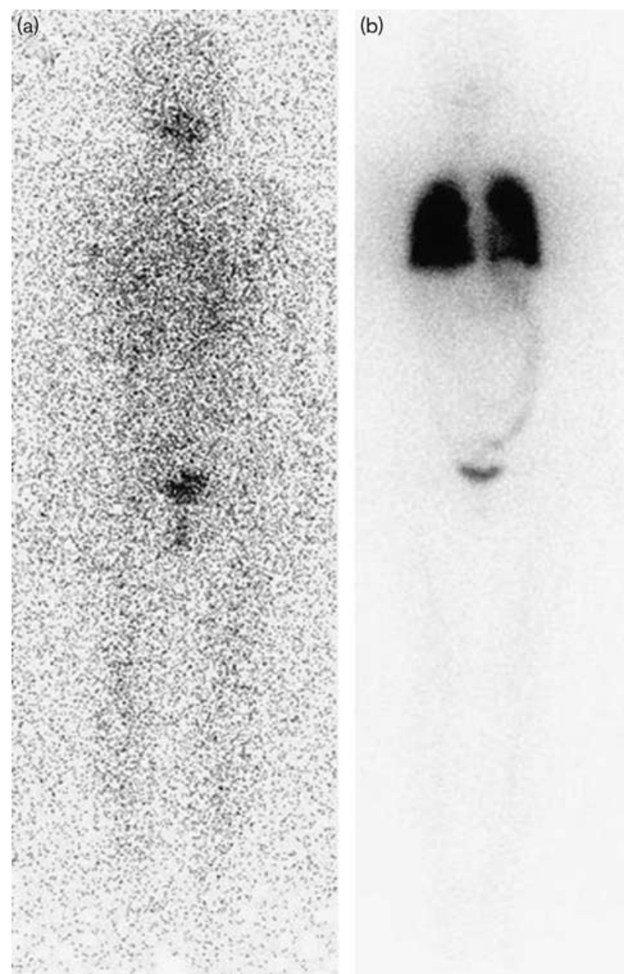
Materials and methods

Patients and serum sample preparation

Blood samples were obtained from PTC patients who had received ^{131}I therapy at the Nuclear Medicine Department of the Sixth People's Hospital affiliated to Shanghai Jiao Tong University, a major ^{131}I treatment center in China. The study was approved by the Institutional Ethics Review Board of our hospital and informed consent was obtained from all patients. Before the start of ^{131}I therapy, thyroxin therapy was withdrawn for 3–4 weeks and patients were placed on a low-iodine diet for 2 weeks. The patients included in the study were divided into two age-matched and sex-matched groups: a non-^{131}I-avid lung metastases group (group A) and a ^{131}I-avid group (group B). The criteria for inclusion were a negative history of other tumors, prior total thyroidectomy for PTC without a history of ^{131}I therapy, and the presence of cervical lymph node metastases without any other distant metastases except to the lung. The diagnosis of lung metastases was made on the basis of the presence of diffuse bilateral pulmonary nodules confirmed by a computed tomography scan with significantly increased serum thyroglobulin (Tg) levels.

The post-therapeutic ^{131}I-WBS was performed 3–5 days after the administration of ^{131}I at a dosage of 150–200 mCi. Patients showing no ^{131}I uptake (defined as a level of ^{131}I uptake in the lung that was similar to that in the upper limbs) were placed in group A. Conversely, patients with diffuse ^{131}I uptake in the lung in the ^{131}I-WBS were placed in group B (Fig. 1).

Fig. 1



Typical examples of non-^{131}I-avid lung metastases (a) and ^{131}I-avid lung metastases of papillary thyroid carcinoma after ^{131}I-WBS (b).

Blood samples (5.0 ml) were drawn from 59 consecutive PTC patients with diffuse bilateral pulmonary metastases before the first ^{131}I therapy session. The blood was allowed to clot at room temperature for 15 min. Serum was separated by centrifugation at 3000 rpm for 10 min and stored at -80°C until analysis. According to the inclusion criteria and results of post-therapeutic ^{131}I-WBS, 10 serum samples were placed in group A and 10 in group B. The clinical and demographic data are summarized in Table 1. There were no statistically significant differences between the patients of groups A and B in relation to clinical and demographic parameters.

Removal of high-abundance proteins from serum samples

Serum samples were processed using an Agilent Multiple Affinity Removal Column (Agilent Technologies, Palo Alto, California, USA) according to the manufacturer's instructions in order to remove high-abundance proteins (albumin, IgG, IgA, antitrypsin, transferring, haptoglobin, etc.). Approximately 90% of total serum protein was removed using this method. For each sample, a low-abundance fraction was collected and buffer-exchanged with 10 mmol/l Tris-HCl (pH 7.4) using 5000 Da molecular weight (MW) cutoff spin concentrators (Agilent Technologies). Protein quantification was performed using the Coomassie (Bradford) Protein Assay Reagent kit (Pierce Biotechnology, Rockford, Illinois, USA) and absorbance was measured at 595 nm. Bovine gamma globulin (Sigma, Atlanta, Georgia, USA) was used as a protein standard.

Two-dimensional electrophoresis and imaging

Serum samples from the two groups were homogenized under liquid nitrogen and suspended in lysis buffer (9.5 mol/l urea, 4% CHAPS, 65 mmol/l DTT, and 0.5% carrier ampholyte) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The suspension was homogenized and sonicated on ice and centrifuged at 14 000 rpm for 1 h at 4°C. The supernatant was collected and protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, California, USA).

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Table 1 Clinical and demographic features of group A and group B in this study

	Group A	Group B
Number of patients	10	10
Mean age (years)	41.00±13.81	43.50±13.53
Sex (male/female)	5/5	5/5
Histological diagnosis	PTC	PTC
Prior radioiodine treatment	No	No
Results of CT scan of the lung	Diffuse small nodules	Diffuse small nodules
Serum Tg level (ng/ml)	1975.19±1700.65	2294.50±1614.63
Serum TgAb level (IU/ml)	31.13±30.28	52.11±53.66
^{131}I-WBS results	^{131}I uptake close to upper limbs	Diffuse lung uptake
Extent of disease	No other distant metastasis	No other distant metastasis
Cancer stage	$T_{1-2}N_1M_1$	$T_{1-2}N_1M_1$
Time after surgery (mean)	7.33±2.51 weeks	7.10±2.76 weeks
Previous treatment other than ^{131}I	No	No
Smoking status	No smoking	No smoking
TSH mean level at the time of ^{131}I scan	113.07±29.11 mIU/l	110.80±32.58 mIU/l
Administered doses of ^{131}I	150–200 mCi	150–200 mCi

CT, computed tomography; PTC, papillary thyroid carcinoma.

Proteins were focused on 13 cm (pH 3–10) IPG strips (GE Healthcare, Piscataway, New Jersey, USA) using the IPGphor Isoelectric Focusing system (Amersham Biosciences, Hercules, California, USA). IPG strips were equilibrated in equilibration buffer (50 mmol/l Tris-HCl, 6 mol/l urea, 30% glycerol, 2% SDS) supplemented with 1% DTT to maintain the protein in a reduced state, followed by 2.5% iodoacetamide to prevent reoxidation of thiol groups during electrophoresis. Second-dimension separation was performed using hand-cast 12.5% gels (Amersham Biosciences) at 15 mA/gel for 30 min, and then 30 mA/gel was used until the bromophenol blue was 0.5 cm from the edge of the gel. Samples were run in triplicate. The gels were scanned using a GS-710 imaging densitometer (GE Healthcare), and protein spots that were differentially expressed between the two groups were selected for identification.

MALDI-TOF/TOF mass spectrometry and peptide mass fingerprinting analysis of differentially expressed protein spots

Differentially expressed protein spots were excised from the gel, destained, and washed. Gel pieces were incubated in 0.2 mol/l NH_4HCO_3 for 20 min and then lyophilized. Each spot was digested overnight in 12.5 ng/ml trypsin in 0.1 mol/l NH_4HCO_3, and the peptides were extracted three times with 60% acetonitrile (ACN, CH_3CN), 0.1% trifluoroacetic acid (TFA). Peptide mixtures were analyzed using MALDI-TOF/TOF (4700 proteomics Analyzer; Applied Biosystems, Foster City, California, USA). MS/MS data were acquired with a N_2 laser at a sampling rate of 25 Hz. Peptide mass fingerprinting (PMF) and mass spectrometry (MS) data were combined using Flex analysis and the combined data sets were submitted to MASCOT for protein identification. A search of the National Center for Biotechnology information (NCBI) nonredundant database was conducted. Search parameters were the enzyme trypsin, one missed cleavage, fixed modification of carbamidomethyl (C), and variable modification of oxidation (Met). A peptide tolerance of ±100 ppm, a fragment mass tolerance of ±0.4 Da, and a peptide charge of +1 were selected. Protein scores CI% > 95 were considered statistically significant ($P < 0.05$) under the above parameters. The experiment was repeated three times, and the selected criteria were protein spots showing expression changes of at least 1.5-fold between the two groups when compared in terms of average relative volume, reproducible spots detected in three replicate experiments, and successful identification of spots using MALDI-TOF/TOF analysis.

Screening proteins of interest by bioinformatics analysis

The IPI-Human v3.53 protein database and the related gene information were used to estimate protein residues, isoelectric points, and molecular weights with the EMBOSS-PEPSTATS tool. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses were performed on the differentially expressed proteins. Important and key proteins were selected for further study.

Validation of proteins of interest by western blotting analysis

To further validate the mass spectrometry results, serum samples from an additional 25 PTC patients with non-^{131}I-avid lung metastases and 25 PTC patients with ^{131}I-avid lung metastases were collected for western blot analysis. Total protein lysates (20 μg) were electrophoresed on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were incubated overnight with primary antibody (diluted 1.25 μg/ml; Sigma) at 4°C. After washing with TBST three times, the membranes were further incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG; Santa Cruz, Santa Cruz, California, USA), diluted 1 : 10 000 in blocking buffer for 1 h at room temperature and then washed three times with TBST. Immunoreactions were visualized using the enhanced chemiluminescence system (ECL+; GE Healthcare). Experiments were repeated three times.

Results

Differentially expressed proteins between group A and group B

More than 100 protein spots were detected by 2-DE differential protein analysis. Twenty-seven differentially expressed proteins were selected on the basis of an expression difference greater than or equal to 1.5-fold between the two groups, as determined using biological variation analysis and a module of DeCyder software (GE Healthcare) (Fig. 2). However, spots 2256 and 2343 could not be picked up. Of the remaining 25 protein

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spots, 14 were found to be significantly upregulated and 11 were significantly downregulated in group A.

Identification of differentially expressed proteins by MALDI-TOF/TOF MS

The 25 protein spots were excised from the gels, treated by trypsin digestion, and submitted for identification. Twenty-four protein spots were identified successfully, whereas one spot (1985) could not be identified by MALDI-TOF/TOF MS, probably because the amount of protein was too low. Six protein spots (748, 1371, 1433, 1943, 1752, and 1821) could not be matched to proteins in the NCBI database. The remaining 18 spots corresponded to 11 proteins identified by MS and PMF analyses (Table 2). The representative MS and MS/MS results for afamin are shown in Figs 3 and 4.

Of the 11 identified proteins, six were downregulated [α-1B-glycoprotein (A1BG), carboxypeptidase *N*-polypeptide 1, complement factor H-related 1 (CFHR1), group-specific component, inter-α (globulin) inhibitor H_2 (ITIH2), and afamin] and five were upregulated [histidine-rich glycoprotein, serum amyloid P component, cp 20 kDa protein, complement component C7, and keratin, type I cytoskeletal 9 (KRT9)] in group A.

Bioinformatics analysis of differentially expressed proteins

The 11 proteins identified were further analyzed using a bioinformatics approach to filter key proteins that were most relevant to this study. The results of the analysis are as follows: one was a cytoskeletal protein (KRT9), which is a component of the supporting structure of the cell and the attachment bracket of cytoplasmic components. Three were immune response proteins (C7, CFHR1, and ITIH2). Another was carboxypeptidase N, which is a plasma metalloprotease that cleaves basic amino acids from the C terminal of peptides and proteins. The enzyme is important in the regulation of peptides such as kinins and anaphylatoxins. One was a histidine-rich glycoprotein (HRG), which is a plasma glycoprotein with a multidomain structure that mainly modulates the immune, vascular, and coagulation systems. There was a serum amyloid P component, which is a nonfibrillar glycoprotein belonging to the pentraxin family of the innate immune system. It plays an important role in amyloid plaque formation and stability. The group-specific component (GC) is the main vitamin D-binding protein in plasma. The Cp 20 kDa protein is known by its exact molecular weight. Finally, A1BG and afamin, which are carcinogenesis-related proteins, were selected for further validation by western blotting.

Validation of differentially expressed proteins of interest by western blotting

Downregulation of serum A1BG and afamin in non-^{131}I-avid lung metastases of PTC was validated in an independent set of serum samples (serum samples from 25 PTC patients with ^{131}I-avid lung metastases and 25 PTC patients with non-^{131}I-avid lung metastases) by western blotting to evaluate their potential as serum biomarkers. The characteristics of the patients used for independent validation were matched with those of the experimental group patients. The protein levels of afamin were significantly lower in the serum samples from group A compared with those from group B (Fig. 5), which correlated with the 2-DE results. However, the protein levels of A1BG did not differ significantly between the two groups.

Discussion

^{131}I therapy is effective in the treatment of postoperative recurrence and metastases of DTC [7]. ^{131}I therapy plays dual roles in the diagnosis and treatment of DTC, which is important to improve the quality of life and prolong survival, in particular in patients with metastatic thyroid cancer [8]. However, the significant uptake of ^{131}I in metastases is a prerequisite for achieving favorable therapeutic effects. It is not recommended for those with non-^{131}I-avid metastasis, and this functional change adversely affects prognosis [2].

Proteomics has the potential to redefine the management of cancers by facilitating the comprehensive identification of biomarkers for screening and diagnosis and the detection of novel therapeutic targets [9]. Although used extensively in other forms of malignancy, the proteomic approach has had limited application in studies on DTC [10–12]. An early proteomic study [13] performed on pooled protein extracts identified S100A6, a Ca^{2+}-binding protein belonging to the S100 family, to be overexpressed in PTC when compared with matched normal thyroid tissue. A more recent proteomic study [14], which included SELDI-TOF-MS, western blotting analysis, and IHC, similarly revealed a significantly higher expression of S100A6 in PTC in comparison with follicular thyroid carcinoma (FTC) or normal thyroid tissue. Srisomsap *et al.* [15] focused on the differential protein expression between non-neoplastic and neoplastic thyroid disease, and the most distinctive protein found was cathepsin B. Netea-Maier *et al.* [16] applied proteomics and IHC tools to identify and confirm potential novel biomarkers that may help distinguish between FTC and follicular thyroid adenoma.

The above proteomic studies focused on the identification of differentially expressed proteins in benign and malignant thyroid cancer. However, no proteomic studies on ^{131}I treatment of DTC metastases have been reported so far. In the present study, we adopted 2-DE for protein separation and PMF by means of MALDI-TOF/TOF MS for protein identification. As an analytical technique, 2-DE is useful for the detection of intact and fragmented protein species, in addition to providing information on post-translational modifications [17]. After MALDI-TOF/TOF

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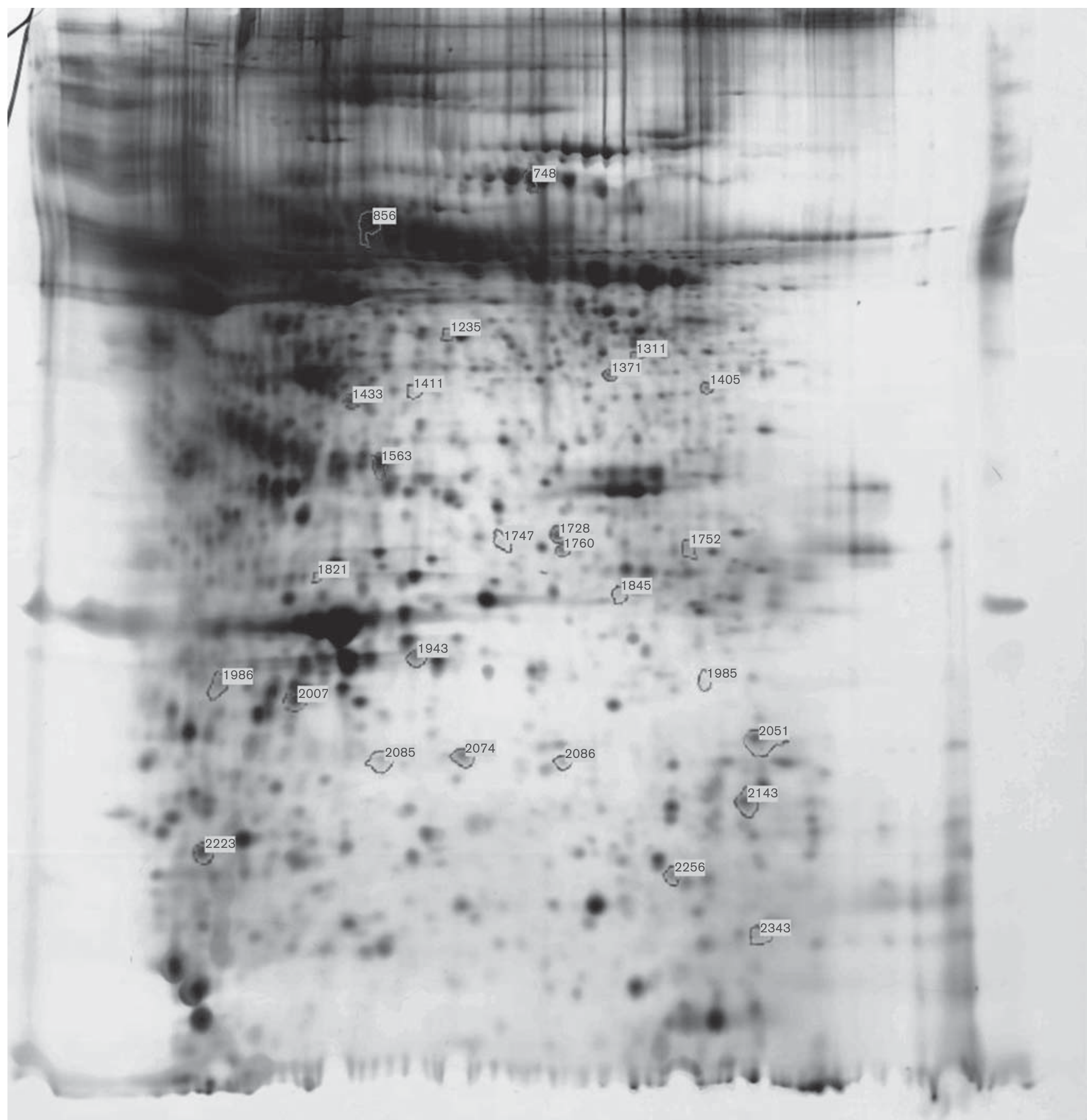
Fig. 2



Differentially expressed protein spots identified by 2-DE analysis in the ^{131}I-avid group. Spots marked by circles correspond to the identified proteins listed in Table 2. Twenty-five spots that showed significant changes were selected for MALDI-TOF/TOF MS analyses. Spots 2256 and 2343 could not be picked up.

MS and PMF identification and bioinformatics analysis, A1BG and afamin were selected for further independent validation by western blotting. Finally, afamin was confirmed as being downregulated in the serum of PTC patients with non-^{131}I-avid lung metastases by western blotting.

A1BG, a protein of unknown function present in human plasma, consists of a single polypeptide chain N-linked to four glucosamine oligosaccharides. It appears to have evolved from an ancestral gene with homology to the immunoglobulin supergene family [18]. In a recent study, A1BG was found to be differentially expressed in

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Table 2 Differentially expressed proteins identified by MALDI-TOF/TOF between group A and group B

Spots No.	Accession No.	Protein name	MW	pI	Protein score	Protein score CI%	Fold change (A/B)
S856	IPI00022895	α-1B-glycoprotein, A1BG	52384.6	6.55	149	100	−2.16
S1235, S2086	IPI00022371	Histidine-rich glycoprotein, HRG	60510.2	7.09	261	100	2.54
S1311	IPI00010295	Carboxypeptidase N, polypeptide 1, CPN1	52538.4	6.86	152	100	−2.39
S1405, S2143	IPI00011264	Complement factor H-related 1, CFHR1	38766.4	7.38	212	100	−2.88
S1411	IPI00022391	Serum amyloid P component, ACPS	25485.2	6.1	454	100	2.10
S1563, S2007, S1760	IPI00968182	Group-specific component, GC	39969.3	5.36	673	100	−1.62
S1986	IPI00019943	Afamin, AFM	70962.7	5.64	191	100	−2.78
S2085, S2074	IPI00793108	cp 20 kDa protein	38940.5	6.28	102	100	1.58
S2223, S2051	IPI00645038	Inter-α (globulin) inhibitor H2, ITIH2	105606.2	6.56	95	99.997	−1.73
S1747, S1728	IPI00296608	Complement component C7, C7	96650.5	6.09	174	100	2.01
S1845	IPI00019359	KRT9 Keratin, type I cytoskeletal 9	62254.9	5.14	88	99.987	2.40

Fig. 3

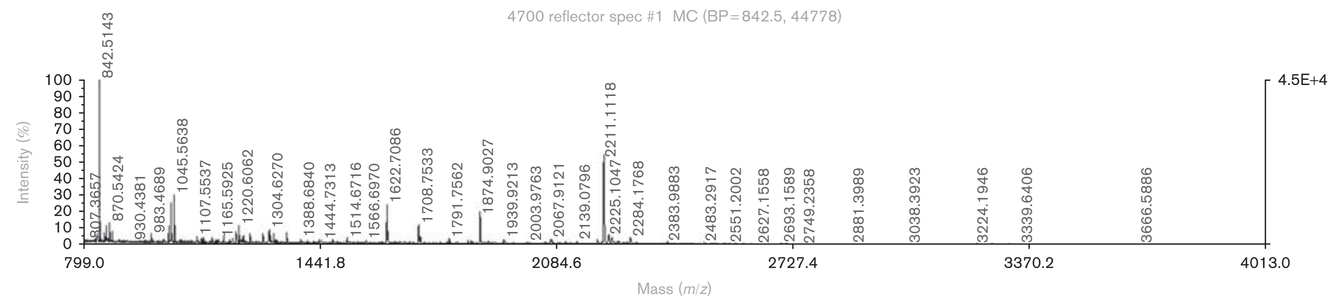
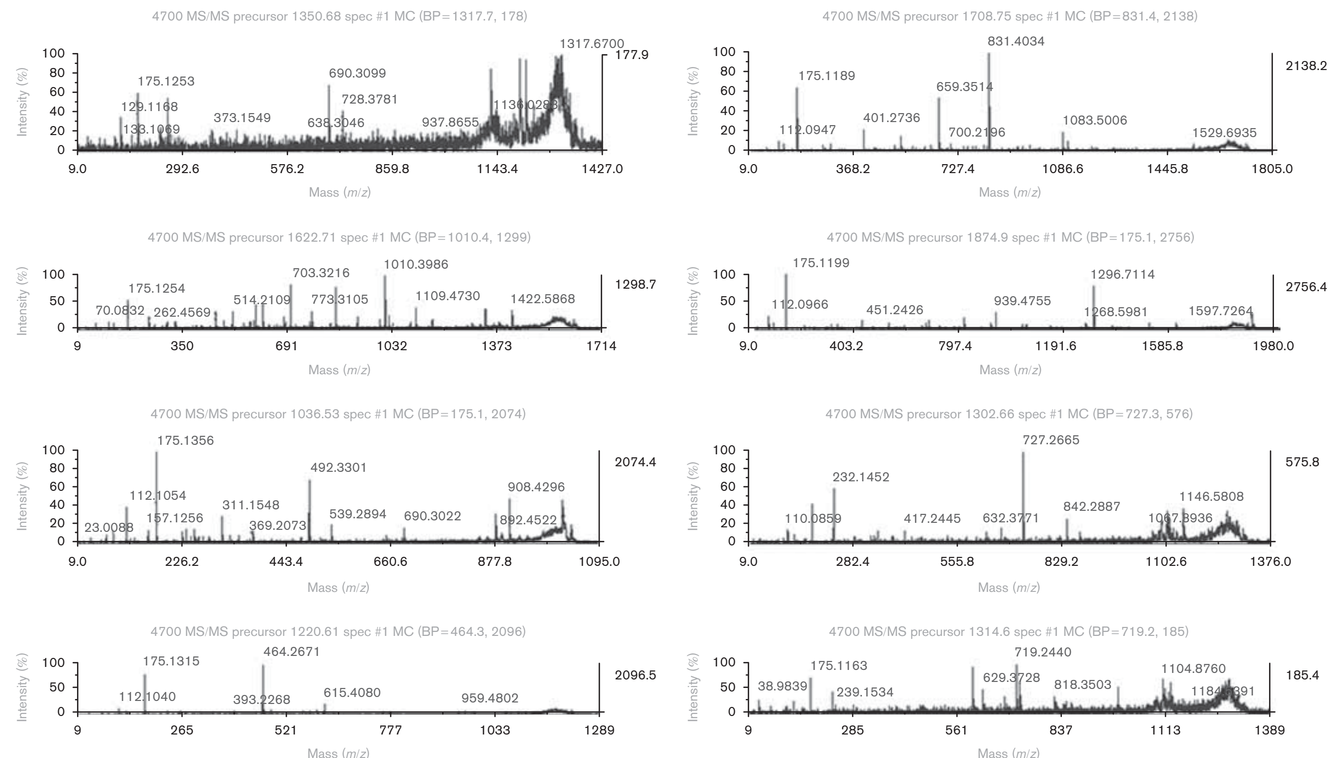


Diagram showing the identification of the representative protein afamin using MALDI-TOF/TOF MS.

Fig. 4



Output of database searches by the MASCOT program performed using MS/MS data for the identification of afamin.

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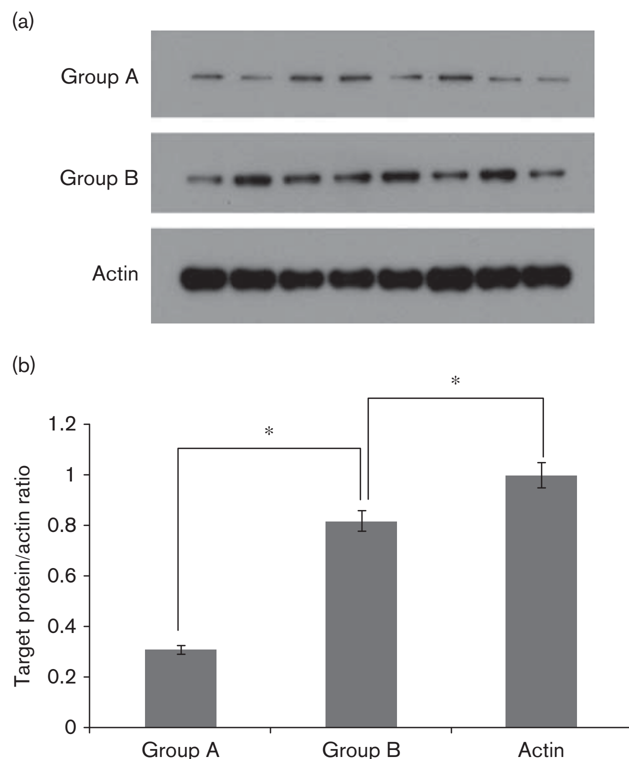
Fig. 5



(a) Western blotting confirmation of the expression differences of the proteomics-identified proteins. Representative blots for afamin, showing differences in protein levels between the two groups (non-^{131}I-avid vs.^{131}I-avid). (b) Quantitative data for afamin. The bar graph depicts the changes in protein expression levels of afamin normalized to the corresponding actin levels. Error bars indicate SD (*$P<0.05$).

pancreatic ductal adenocarcinoma [19] and bladder cancer [20].

Afamin, or α-albumin, is the fourth member of the albumin family, which includes albumin, α-fetoprotein, and vitamin D-binding protein [21]. In contrast to other family members, afamin is highly glycosylated and has a molecular weight of 87 kDa. It is expressed predominantly in the liver and is also present in plasma/serum, cerebrospinal fluid, and follicular fluid [21,22]. Afamin has a binding affinity for both α-tocopherol and γ-tocopherol, two of the most important forms of vitamin E, *in vitro* [23]. Vitamin E has been shown to play a crucial role in the protection against oxidative damage and disease. The thyroid gland is a unique endocrine organ that requires hydrogen peroxide (H_2O_2) for thyroid hormone formation. H_2O_2 production in the thyroid gland is regulated by dual oxidase 2 (DUOX2) [24]. Thyroid cells contain antioxidants to protect them from H_2O_2-mediated oxidative damage, and alterations in this balance may result in thyroid cell dysfunction [25]. Lacroix *et al.* [26] reported that the expression of DUOX proteins was related to thyroid tumor differentiation. Several studies have reported that afamin appeared in the blood of patients with cancer. Jackson *et al.* [27] and Dieplinger *et al.* [28] discovered and validated serum afamin as a potential biomarker for ovarian cancer using proteomic-based approaches. These studies reported that afamin is downregulated in the serum of ovarian cancer patients and speculated on the possible antioxidative properties of afamin. Ovarian cancer is one of the most common types of cancer in women. Interestingly, the incidence, aggressiveness, and death rate of thyroid cancer differ significantly according to sex [29]. DTC incidence rates are also higher among women than among men. Given the substantially higher incidence rates and the proliferative effect of estrogen on thyroid cells [30], a possible hormonal etiology of thyroid cancer is hypothesized.

In the present study, significant differences between the protein expression profiles of PTC patients with ^{131}I-avid lung metastases and those with non-^{131}I-avid lung metastases were detected by proteomic analysis. Afamin was downregulated in the serum of PTC patients with non-^{131}I-avid lung metastases. This is the first report of afamin in human thyroid disease detected by proteomic profiling. This newly identified protein may be helpful for screening PTC patients with ^{131}I-avid lung metastases before ^{131}I therapy. The ability to predict ^{131}I uptake is of clinical value for the design of therapeutic strategies involving radioiodine treatment. Furthermore, our results may provide a basis for understanding the mechanisms leading to the loss of ^{131}I uptake (dedifferentiation) and help identify potential therapeutic targets for the treatment of PTC patients with non-^{131}I-avid metastases. However, the validated sample size in this preliminary study was limited, and a larger number of samples are needed to further confirm our data. Moreover, the specific role of afamin in thyroid carcinoma deserves further investigation. Nevertheless, the present study may serve as a valuable foundation for future studies.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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