High-resolution proteomics and metabolomics in thyroid cancer: Deciphering novel biomarkers

Diana Navas-Carrillo[1] MD, PhD; José Manuel Rodriguez[2] MD, PhD; Silvia Montoro-García[3], PhD; Esteban Orenes-Piñero[4] PhD

[1]Department of Surgery, Hospital de la Vega Lorenzo Guirao, University of Murcia, Murcia, Spain; [2]Department of Surgery, Hospital Universitario Virgen de la Arrixaca, University of Murcia, Murcia, Spain; [3]Cell Culture Lab, Catholic University of Murcia, Murcia, Spain; [4]Proteomic Unit, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca (IMIB-Arrixaca), Universidad de Murcia, Murcia, Spain

Running title: Proteomics and metabolomics in thyroid cancer

Address for corresponding:

Esteban Orenes-Piñero, PhD

Proteomic Unit, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca (IMIB-Arrixaca).

Ctra. Madrid-Cartagena, El Palmar, Murcia, Spain.

Phone: 0034-968-381288. E-mail: eorenes@um.es

Abstract

Thyroid cancer (TC) is the most common endocrine malignancy and its incidence has been increasing sharply since the mid-1990s, being the fastest-increasing cancers in both men and women. Increased medical surveillance, the effect of environmental factors and more sensitive diagnostic tests, such as ultrasound and confirmation via fine-needle aspiration biopsy, are thought to account for this increased incidence. There are several histological types of thyroid cancer, including papillary thyroid carcinoma, follicular thyroid carcinoma, medullary thyroid carcinoma, and anaplastic thyroid carcinoma. Determining the type of thyroid cancer is crucial for the assessment of prognosis and treatment selection. Unfortunately, approximately 20–30% of patients undergoing fine-needle aspiration biopsy have inconclusive or indeterminate results, leading to unnecessary surgical intervention in 80% of patients with benign nodules. To resolve this diagnosis dilemma, new biomarkers of thyroid cancer are needed.

Proteomic approaches offer an unbiased platform for the comprehensive analysis of the whole proteome in a certain physiological time. Although mRNA expression is widely considered to be indicative of protein expression, protein levels are the result of protein synthesis and degradation, and RNA levels are not informative of protein degradation. Clinically, there is increasing evidence for the role of proteomic and metabolomic technologies in biomarker discovery, providing novel information on the molecular events associated with TC, and potentially lead to the identification of novel drug targets. In this review, we will thoroughly describe the importance of novel proteomic and metabolomic approaches to identify new biomarkers associated with TC.

Keywords: Thyroid cancer, proteomics, metabolomics, mass spectrometry, biomarkers.

Abbreviations

2D-DIGE: Two-dimensional differential gel electrophoresis.

ATC: Anaplastic thyroid carcinoma.

BFTA: Benign follicular thyroid adenoma.

FFPE: Formalin-fixed paraffin embedded.

FNAB: Fine-needle aspiration biopsy.

FTC: Follicular thyroid carcinoma.

IHC: Immunohistochemistry.

iTRAQ: Isobaric tags for relative and absolute quantitation.

MTC: Medullary thyroid carcinoma.

NMR: Nuclear magnetic resonance.

NMTC: Non-medullary thyroid cancer.

PTC: Papillary thyroid carcinoma.

ROC: Receiver operating characteristic.

TC: Thyroid cancer.

VDAC1: Voltage-dependent anion channel 1.

Introduction

Thyroid cancer (TC) is a common malignancy with a rapidly increasing global incidence[1]. Although mortality from thyroid cancer is relatively low, the rate of disease recurrence or persistence is high, leading to increased patient morbidity and mortality. Moreover, the American Cancer Society has reported that the incidence of thyroid cancer is increasing more rapidly compared to other cancers[2].

TC is a general term that comprises two main groups of neoplasias, depending on the cell type affected by the malignant transformation. 1) Carcinomas originating from the follicular epithelium, referred to as non-medullary thyroid cancer (NMTC) representing more than 95% of all TC; and 2) carcinomas originating from the parafollicular thyroid C cells, referred to as medullary thyroid cancer (MTC) accounting less than 5% of all TC. There are four histologic subtypes of NMTC: papillary (PTC) (85%), follicular (FTC) (11%), Hürthle cell (3%) and the anaplastic histotype (ATC) (1%)[1]. Histologically, PTCs are composed of well differentiated epithelial cells and can be distinguished by distinctive nuclear alterations including pseudoinclusions, grooves, and chromatin clearing. On the other hand, FTC lacks the morphological nuclear features of PTC. An important histologic variant of FTC is the oncocytic (Hürthle cell, oxyphilic) follicular carcinoma composed of eosinophilic cells repleted with mitochondria[3]. ATCs, the most uncommon form of NMTCs, are characterized by undifferentiated cells with high mitosis rate, necrotic areas, spindle-like cell morphologies as well as giant and occasionally squamous cells.

Clinically, PTC incidence is remarkably high in developed countries, it is typically slow growing, and when it spreads it usually invades neighboring tissues and occasionally metastasizes to regional lymph nodes; whereas FTC often metastasizes to bone and lung. On

the other hand, ATC behaves very aggressively, rapidly invades adjacent tissues and is considered one of the most lethal human cancers[4].

Importantly, FNAB with cytopathological examination is the gold standard for thyroid cancer diagnosis and classification. According to the European Thyroid Association Guidelines[5], in 2–16% of cases cytology is not diagnostic, i.e., the material is insufficient for diagnosis and an FNAB repetition is requested. In 5–20% of cases, moreover, it is not possible to discriminate between benign and malignant nodules because of an indeterminate cytology, including follicular proliferation and atypia of undetermined significance[5]. Only a minority (about 20%) of these indeterminate thyroid nodules has been proved to be malignant at final histology; thus undergoing to unnecessary hemi- or complete thyroidectomy surgical intervention in 80% of patients with benign nodules. Furthermore, these patients are required to take life-long hormone replacement treatment[6]. For these reasons, the discovery of novel TC biomarkers is needed for an early detection, a better classification and an optimum treatment.

Similarly to genomic technologies, proteomic approaches allow the discovery of disease-specific targets and biomarkers, providing promising diagnostic and prognostic information. Proteomics can be defined as 'the knowledge of the structure, function and expression of all proteins in the biochemical or biological contexts of all the organisms'[7] and is an important approach to unraveling pathophysiology and for early detection of disease. The challenge of proteomics versus genomics resides in the complexity of protein chemistry and multiple potential post-translational functional modifications, in contrast to the unique binding of nucleotides on which genomics relies[8]. It can be said that while genomics explains the disposition to disease, proteomics gives a more accurate reflection of exact disease processes at the time of study[9]. The goal of biomarker discovery using proteomic studies in biological

matrices is to identify proteins that are truly differential in abundance between a population of cases and suitable controls, or before and after a relevant perturbation. The technique usually uses label-free methods to analyze individual samples from each class or condition, often few in number, and to employ a differential cutoff to discriminate real differences from technical artifacts or biological noise[10]. Despite the procedure performed, sample preparation, data acquisition and processing, biostatistical analysis, data interpretation and biological validation of "in silico" results, are the general approaches in most proteomic analysis (Figure 1).

On the other hand, metabolites play an important role in understanding disease phenotype and elucidating pathophysiological mechanisms by means of identification of altered biochemical pathways in the system[11]. Human metabolomes are relatively well known, defined, and catalogued. The estimated number of components of human metabolomes ranges from thousands to tens of thousands, depending on type of a cell or tissue[12]. It is widely known that cancer cells reprogram biochemical pathways and alter the metabolism of the cell to supply high energy and biosynthetic needs for cell growth and division. These alterations mainly include increasing of glucose and glutamine uptake, lactate production and biosynthesis of lipids, proteins and nucleic acids[13]. Thus, metabolomics provides valuable information about metabolism of malignant cells and has a great potential in cancer research as well as in identification of novel diagnostic and prognostic markers.

The aim of this review is to describe comprehensively the role of proteomic and metabolomic approaches to identify new biomarkers of TC and provide new information on the molecular events associated with TC and the identification of novel drug targets.

Material and Methods

Published data for this review were identified by search and selection in the MEDLINE database and reference lists from relevant articles and reviews. A two-step approach was used; proteomic and metabolomics biomarkers associated with TC were identified in a search with the keywords “proteomics” “metabolomics” and “thyroid cancer”. Secondly, another search was made using a combination of the words “2D-DIGE”, “mass spectrometry imaging”, “nuclear magnetic resonance spectroscopy” and “thyroid cancer”. Bibliographies of all selected articles and review articles about proteomics and/or TC were reviewed for other relevant articles.

Proteomic and metabolomic approaches in TC

Proteomic and metabolomics techniques are usually classified depending on whether the protein measured is known or unknown when defining the experimental design. Bidimensional gels (2D) and mass/weight spectrometry techniques are ideal approaches for identification purposes. Fluorescent labeling of serum protein extracts using a two-dimensional differential gel electrophoresis (2D-DIGE) strategy has been widely used in TC to decipher novel biomarkers[14-17]. The advantage of 2D-DIGE relies on defining statistical significance on proteins identified differentially expressed between disease and control specimens. 2D-DIGE reduces experimental variability and enhances quantitative precision because two samples (or sample pools) can be compared on a single 2D gel. Consequently, both major and minor differences in expression levels that might be missed by other approaches can be established. As part of the experimental design (Figure 1), 2D-DIGE and mass spectrometry are explored to identify differentially expressed proteins in TC patients compared with paired controls. Besides, identified proteins can be tested to be over or under-

expressed in thyroid tumors samples to analyze their importance in cancer progression. Moreover, antibodies against novel proteins identified in 2D-DIGE and mass spectrometry can be used in a bigger set of patients and controls (Western-blotting, ELISA analyses) to confirm lower or higher expression in tumor samples.

The use of isobaric tags for relative and absolute quantitation (iTRAQ) combined with multidimensional liquid chromatography (LC) and tandem mass spectrometry (MS) analysis[18] is a powerful methodology in the search for disease specific targets and biomarkers using cell lines, tissues, and body fluids (Figure 2). The iTRAQ method places tags on primary amines (NH_2-terminal or -amino group of the lysine side chain), allowing detection of most tryptic peptides. Because of the isobaric mass design of the iTRAQ reagents, differentially labeled peptides appear as single peaks in MS scans, but when subjected to tandem MS (MS/MS) analysis, the mass-balancing carbonyl moiety is released as a neutral loss, thereby liberating isotope-encoded reporter ions that provide relative quantitative information on the proteins from which the peptides originate[19].

Matrix-Assisted Laser Desorption/Ionization (MALDI) and Mass Spectrometry-Imaging (MSI) are new promising technologies that are able to detect several different compounds directly on fresh frozen tissues, on formalin fixed paraffin-embedded specimens, or even in cells[20]. A matrix is applied on cryosectioned tissue and a very small area (as small as >200 μm) of the matrix-applied tissue is analyzed spot by spot using MALDI-MS. Differences between the analyzed areas are displayed by the imaging program (Figure 3). Using all the spectral data from each spot, the magnitudes of specific peaks on each spot can be displayed in terms of color intensity. This way, spatial information on the tissue can be obtained[21]. MALDI-Imaging has the ability to provide very precise and localized information regarding protein expression in cytological specimens[22]. This enables the detection of cancer cell

subpopulations based on their different protein profiles, even within regions that are histologically indistinguishable at the microscopic level[23]. This is of paramount importance in thyroid pathology, as the heterogeneous cellular composition of the thyroid tissue may hamper the biomarker discovery studies. Compared with conventional imaging methods, IMS does not require the additional use of specific antibodies against the protein, and integrates histopathology and protein expression. MALDI imaging has been used to build proteomic signatures of carcinoma in different organs such as esophagus, breast, colon, liver, stomach, and thyroid gland[24]. Moreover, MALDI-MSI has been also applied to compare normal and tumor thyroid tissues for biomarker discovery[24].

Regarding metabolomics, the most utilized techniques includes mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. These two techniques have both their advantages and limitations. NMR is generally accepted as the gold standard in metabolite structural elucidation, due to its high selectivity, analytical reproducibility, non-destructive nature, and simplicity of sample preparation[25]; however, it has lower sensitivity compared to MS. NMR spectroscopy enables identification of small biomolecules measuring intrinsic magnetic properties of atomic nuclei, such as ^{1}H, ^{13}C, and ^{31}P, which are commonly present in metabolites[26]. On the other hand, MS is more sensitive, has a higher throughput, depth of coverage and can measure more molecules in a complex biological sample, based on the mass/charge ratio of the analyte ions generated in the spectrometer[27]. Additionally, MS allows the access to large databases for automatic identification of metabolites[28]. High resolution MS experiments with sequential fragmentation of the analyte ions give structural information about the studied compounds[12]; however, it has the drawback that not all metabolites can be ionized. Direct sample injection on high-resolution MS is an effective way to maximize analytical throughput, since sample preparation and measurements are

time-intensive and difficult to automate[29]. Due to its high sensitivity, wide range of molecules that can be analyzed, and the capacity of separating complex mixture of compounds, which is a typical problem in the case of biological materials, the usage of liquid chromatography coupled with MS has expanded rapidly over the past ten years[30].

2D-DIGE in thyroid cancer

As far as we know, one of the first studies analyzing different protein expression in TC using 2D-DIGE technology was performed between PTC samples from women aged 25–50 years with no evidence of chronic lymphocytic thyroiditis, matched normal thyroid tissue (from the same PTC patients, as primary control) and normal tissue from patients with benign adenomas, as secondary control[14]. DeCyder analysis of pooled protein from thyroid tissue (PTC and matched normal) of five different patients with similar clinical characteristics determined that 58 protein spots had at least two-fold volume ratio different expression. When the isoforms of thyroglobulin, a well-known marker of thyroid cancer, were excluded, 31 distinct protein spots were identified. These data were validated by performing individual patient gel analyses, confirming that changes observed in the pooled gel analysis were statistically significant changes for 48% of the identified proteins, thus refining the list of potential biomarkers for further validation strategies. The robustness of this approach was confirmed by its ability to detect previously identified and well-defined PTC markers, such as galectin-3, cytokeratin 19 and cathepsin[31]. Importantly, three novel PTC biomarkers, S100A6, peroxiredoxin 2, and Heat-shock protein-70 (HSP-70) were identified. S100A6 was validated using immunohistochemistry, showing a sensitivity and specificity of 85% and 69%, respectively, in distinguishing benign from malignant lesions. In addition, two distinct isoforms (different pI) of cathepsin B were also found differentially

expressed between normal and cancer tissues. Cathepsin B is a lysosomal cysteine proteinase which is involved in the processing of thyroglobulin in the thyroid[32] and is believed to play a role in tumor invasion and metastasis as it has been previously found over-expressed in many tumors[33]. Interestingly, one form has five-fold lower expression in PTC, while a more acidic form has 2.5–4-fold higher expression in PTC, indicating that post-translational modification may be more important in PTC than total cathepsin B content. Several mitochondrial proteins, such as aconitase 2, ATP synthase and voltage-dependent anion channel 1 (VDAC1) showed also increased expression in PTC when compared to control tissues[14].

A later study, aimed to distinguish between benign follicular thyroid adenoma (BFTA) and FTC by 2D-DIGE approach, as it not possible only using cytological features[15]. Protein extracts from five patients with FTC (some of them with extensive capsular and vascular invasion) were compared with six patients with BFTA. Fifty-four protein spots showed statistically significant ($p<0.05$) abundance differences between individual FTC samples and the BFTA pool. Eleven proteins were excluded from further analysis: that was, albumin, β-globin, thyroglobulin (four distinct spots), and five spots that were identified as a mixture of several proteins. This left 43 protein spots for further consideration (corresponding to 37 distinct proteins). Of them, Heat Shock Protein glycoprotein 96 (HSP gp96), protein disulfide isomerase A3 (PDI A3), and calreticulin were selected for further validation with immunohistochemistry as they were very abundant in tissue samples and showed a large difference in volume ratios between the FTC and FTA. Moreover, the presence of other proteins previously associated with follicular-derived thyroid neoplasia, such as cytokeratins 7, 8, 18 and nucleoside diphosphate kinase-1 (also known as nm23-H1), confirmed the *in-silico* results[34]. Importantly, the intensity scores for immunohistochemical staining

correlated with disease severity and all three proteins showed a high sensitivity with respect to detection of widely invasive FTCs. The combination of all these markers showed a positive predictive value of 75% and a negative predictive value of 68%[15].

More recently, 2D-DIGE coupled with MS was performed in human thyroid tumors and normal samples obtained from frozen tissues in the following groups: normal thyroid tissue (NTT) (n = 15), PTC (n = 5), FTC (n = 4), ATC (n = 3), and adenomas (ADE) (n = 4) to identify possible TC biomarkers that could be used as therapeutic targets in thyroid tumors[16]. Among them, mitochondrial proteins were proposed to play an important role in the carcinogenesis process in epithelial thyroid carcinoma[35]. The analysis of 150 spots identified 102 proteins and 41% of these proteins were involved in mitochondrial metabolism. Six of them: apoptosis inducing factor mitochondrion-associated 1 (AIFM1), 3-hydroxyisobutyryl-CoA hydrolase mitochondrial (HIBCH), peroxiredoxin 3 (PDRX3), peroxiredoxin 5 (PDRX5), ubiquinolcytochrome c reductase core protein II (UQCRC2) and voltage-dependent anion channel 2, (VDAC2) were further investigated. The spot corresponding to VDAC2 protein presented significant expression changes in PTC and FTC compared with normal thyroid tissue (2.2 fold-change; p=0.01). In addition, Bcl-2 antagonist/killer-1 (BAK1) and Bcl-2-associated X protein (BAX), both required to initiate the apoptosis mitochondrial pathway, showed the strongest relationship with VDAC2. It has been proposed that VDAC2 gene could regulate BAK1 activation through interaction with the hydrophobic tail of BAK, promoting inhibition of the mitochondrial apoptotic pathway[36]. A double-validation by qRT-PCR and expression in two thyroid tumoral cell lines, TPC-1 (4.2-fold-change, p=0.001) and CAL-62 (5.3-fold-change, p=0.015) confirmed the over-expression of VDAC2 observed in the *in-silico* results confirming its role in resistance to apoptosis previously observed in neoplastic thyroid cells[37].

In another study, 2D gels and MS technologies were used to identify novel biomarkers for TC diagnosis and more importantly, to differentiate between PTC and FTC[17]. Analyses were carried out in papillary and follicular thyroid carcinoma cell lines, and the biomarkers were validated in five PTC and five FTC tissues, with their adjacent normal tissues from Thai patients. Eight biomarkers could distinguish PTC from normal tissues, namely enolase 1, triose phosphate isomerase (TPI), cathepsin D, annexin A2, cofilin 1, proliferating cell nuclear antigen (PCNA), copine 1 and heat shock protein 27 kDa (HSP27); whereas all of them except Annexin A2 can also discriminate FTC from normal tissues. Of note, annexin A2, cofilin 1, PCNA and HSP27 can be used to distinguish between PTC and FTC. Moreover, p53 might be an additional biomarker to indicate the aggressiveness of thyroid carcinomas[17].

All these studies demonstrate the importance of 2D-DIGE approach combined with MS to identify potential novel biomarkers in TC. However, further studies using a greater number of samples, samples from less invasive procedures and/or from various ethnic populations are needed to confirm the usefulness of these biomarkers for clinical diagnosis. Moreover, these potential biomarkers could be used in multi-marker panel for better diagnosis, early detection and classification of TC.

iTRAQ analysis in thyroid cancer

Newer proteomic approaches have enabled deeper quantitative analyses revealing expression of thousands of proteins, improving our understanding in disease biology and proposing new markers of diagnostic potential. A recent study aimed to identify diagnostic markers for discrimination between the cystic variant of PTC (cPTC) and benign thyroid

cystic lesions through the investigation of the fluid accumulated by these lesions using iTRAQ technology[38]. In this study, 20 cPTCs together with 56 benign cystic thyroid lesions were analyzed and 41 proteins were differentially expressed in a significant manner ($p<0.05$). Additionally, 10 of these proteins were also differently expressed between the cPTCs and benign lesions using a more stringent p-value of 0.01. Four proteins that were found up-regulated in cPTCs using in-silico approaches were then selected for validation by immunohistochemistry (IHC), Western blot and ELISA analysis: cytokeratin 19 (CK-19), S100A13, annexin A3 (ANXA3) and carboxymethylenebutenolidase homolog (CMBL). ROC curves analyses determined 55 ng/ml or more as the optimal cut-off for CK-19 concentration in cyst fluid for discrimination of cPTC from benign thyroid cysts by ELISA. Moreover, this suggested cut-off value was consistent with receiver operating characteristic (ROC) curve parameters corresponding to a level of good diagnostic accuracy, supporting the role of CK-19 in diagnosis of cPTC. These results indicate that CK-19 levels in cyst fluid can be an effective complementary tool to routine FNAB for discrimination of cPTC from benign thyroid cysts, which is in agreement with previous meta-analysis suggesting CK-19 as a marker of thyroid malignancy[39]. Regarding S100A13, ROC curve analyses determined the concentration >230 pg/ml as the optimal cut-off in cyst fluid for discrimination of cPTC from benign thyroid cysts by ELISA. However, a poor diagnostic utility of S100A13 was obtained[38]. Both, ANXA3 and CMBL were not confirmed after validation analysis.

Importantly, iTRAQ analysis have not been only performed to identify potential novel biomarkers in TC, as it has also been used to validate MS results in several fresh frozen thyroid tissues from normal histological samples, follicular adenoma (FA) tissues, minimally invasive FTC and classic PTC[6]. Hierarchical cluster analysis of protein

expression based on ANOVA showed a clear separation of PTC tumors from all other sample groups and marked similarity between FA and FTC tumors. Both follicular tumor types, FA and FTC, showed marked reduction (>10-fold) of the extracellular tumor suppressor proteoglycan decorin, also known as PGS2, a negative regulator of receptor tyrosine kinases and growth factor signaling[6]. Decorin has been previously recognized as a multifaceted anti-oncogenic protein of the tumour microenvironment, regulating key cellular functions including proliferation, survival and migration[40], therefore, it could be considered an attractive therapeutic target that could also be useful in thyroid tumours of follicular histology. Additionally, FTC showed altered expression of other extracellular proteins that have been previously associated with tumourigenesis, such as collagen alpha-1 (XV) chain, serpin H1 and tenascin[41,42]. Furthermore, the comparison of FA with FTC tumors revealed that the 72 kDa extracellular protein TGFβ-induced protein ig-h3 (TGFBI) could correctly differentiate most FTC samples from the FA group with a relatively high fold change[6]. This protein could be involved in the progression from thyroid adenoma to carcinoma being a novel candidate marker of malignancy in follicular thyroid tumors. The fact that the expression of most proteins remained unchanged between FA and FTC is a clear demonstration of the high similarity of the molecular phenotype of these two histotypes and underscores why it has been difficult to obtain a molecular signature of malignancy among tumors with follicular histology[6]. These studies confirm the role of iTRAQ technology combined with high-resolution MS as a novel approach to identify potential novel biomarkers and to differentiate between follicular TC histotypes, otherwise indistinguishable by FNAB cytology.

Imaging Mass Spectrometry in thyroid cancer

Recent progress in imaging mass spectrometry (IMS) has made it possible to identify several cellular components such as proteins, drugs, and other endogenous molecules directly on tissue sections[43]. As far as we know, the first study using MALDI-IMS was performed in 2012 to generate proteomic signatures for 6 different tumors (thyroid tumor among them) and could discriminate between different tumor types at different organ sites[44]. Later, proteomic screening by MALDI-IMS was performed in 29 PTC patients to identify protein biomarkers that could distinguish patients with aggresive tumor behavior[45]. Thioredoxin, S100-A10 and S100-A6 were found differentially expressed between metastatic and non-metastatic tumors and validation analyses using IHC found that the overexpression of these three proteins was highly associated with lymph node metastasis in PTC ($p < 0.005$). More recently, fresh frozen tumor samples from 5 PTC patients undergone total thyroidectomy were analyzed using this technology, finding ribosomal protein P2 associated with TC with high confidence levels[21].

Importantly, MALDI-IMS can be also performed in tissue microarrays from formalin-fixed paraffin embedded (FFPE) samples as demonstrated in a series of 44 samples including hyperplastic nodules (HP), follicular adenomas (FA) and PTC[46]. Of note, FFPE tissue samples for proteomic investigations assures high statistical power for the discovery of possible new diagnostic markers and pathological characterization of neoplasms, due to the high number of cases analyzable and the easier recruitment of FFPE samples compared to fresh frozen tissues. In this study[46], fibronectin (FINC), Cytoplasmic Actin 1 (ACTB1), Prelamin A/C (LMNA), Heat Shock cognate 71KDa Protein (HSP7C) and Adenylate Cyclase Isoenzyme 1 (KAD1) appear to be upregulated specifically in the neoplastic tissues, thus representing potential markers of malignancy. A subsequent supervised statistical analysis showed an overexpression of several peptides, such as galectin-3, annexin A1 and

cathepsin-B, among others, in specific areas of malignant characteristics[46]. This finding confirmed the robustness of the *in-silico* analysis as the role of these proteins in thyroid tumors are widely reported in the literature and have been previously discussed in this review.

MALDI-IMS has been also tested in samples from FNAB, the gold standard for thyroid cancer diagnosis and classification. In a very recent study, 13 FNAB from benign thyroid nodules and 13 PTC were used as a training dataset to build and evaluate the discriminatory capability of the MALDI-IMS approach[47]. Then, 10 indeterminate lesions after cytological diagnosis were used in the second phase to verify the discriminatory capability when attempting to classify patients with an unknown histological diagnosis. MALDI-IMS was able to correctly assign, in blind, the specimens to a malignant or benign class, as later confirmed by the morphological classification. Furthermore, MALDI-IMS identified phosphatidylethanolamine-binding protein 1 (PEBP1) and heterogeneous nuclear ribonucleoproteins A2/B1 (ROA2 or hnRNP A2/B1) as potential biomarkers of TC[46]. An over-expression of the PEBP1 in thyroid carcinomas was already shown using 2D-DIGE and MALDI-TOF followed by immunohistochemical validation in a cohort of 121 PTCs[48]. Additionally, an overexpression of hnRNP A2/B1 has been previously reported in lung cancer[49].

All these observations confirm the importance of the novel high-resolution technology MALDI-IMS to identify new potential biomarkers of TC and its discriminatory power to better classify indeterminate lesions not well assigned by FNAB and cytopathological examination.

Metabolomic analyses in thyroid cancer

As a result of the novelty of these techniques, metabolomic studies concerning TC are currently limited to relatively few papers and most of them have been performed on tissue specimens, while studies on biological fluids like serum, plasma or urine are very scarce. Due to extremely diverse physicochemical properties of different metabolites and highly dynamic changes in the composition of the metabolomes, there is no single analytical method allowing examination of the entire metabolome. Regardless of the chosen analytical approach, there are several general steps in the metabolomics studies, including sample collection and preparation, data acquisition and processing, biostatistical analysis, and data interpretation. In order to obtain reproducible results that could be compared between laboratories, strict compliance with the standardized procedures of metabolomic analysis is required[12].

As far as we know, the first metabolomic study in TC was performed using proton magnetic resonance spectroscopy (^{1}H-MRS) of thyroid tissues to discriminate two groups of benign and malignant (papillary, anaplastic, medullary and follicular) thyroid neoplasms from fifty-three patients undergoing partial or total thyroidectomy[50]. The main spectrum differences in papillary carcinoma group included the presence of free and bound amino acids, such as glutamic acid/glutathione, threonine, valine, taurine and bound fucose and absence of lipids compared to healthy controls[50]. Shortly afterwards, the same group analyzed the importance of lipidomics in TC specimens from 93 consecutive thyroid nodules using 2D ^{1}H-MRS, showing a progressive increase in lipid cross peaks assigned to di-/triglycerides when comparing colloid/hyperplastic nodules to follicular adenoma, and adenoma to carcinoma[51]. The potential of NMR techniques in the analysis of lipid component of thyroid cancer was also reported in another study[52]. ^{1}H-NMR was used to quantify lipid extracts of four types of

thyroid tissue [normal (n=27), papillary cancer (n=15), adenoma (n=13) and Basedow disease (n=6)][52]. Dolichol concentrations were significantly lower in papillary cancer and Basedow disease compared to normal thyroid ($p<0.01$ and $p<0.05$, respectively), while cholesterol was enhanced only in cancer tissue. Besides, benign adenoma exhibited normal levels of both dolichol and cholesterol, suggesting that the isoprenoids levels are normal in adenoma but not in cancer[52]. More recently, several studies have also applied novel high-resolution NMR techniques to distinguish between benign and malignant thyroid nodules[53,54]. In one of them, specimens from 72 patients undergoing a total thyroidectomy were analyzed with the previous diagnosis: 28 PTC, 40 indeterminate follicular lesions, and 4 benign nodules. Normal and neoplastic thyroid tissues, as well as benign and malignant neoplasms could be discriminated from each other by elevated levels of several amino acids (mainly phenylalanine and taurine) combined with decreased levels of choline, phosphocholine, myo-inositol, and scyllo-inositol in malignant samples. Additionally, a receiver operating characteristic curve analysis revealed that 77% of the samples were accurately predicted[53]. In another study, aqueous tissue extracts of healthy thyroid tissue (n=19) and thyroid lesions (n=45) comprising non-neoplastic nodules, follicular adenomas (FA) and malignant TC, were analyzed[54]. A clear discrimination was found not only between healthy thyroid tissue and pathological thyroid tissue but also between different types of thyroid lesions. A total of 28 metabolites common to all thyroid lesions were identified, belonging to few biochemical groups: amino acids, organic acids, lipids and carbohydrates. Metabolic changes in thyroid cancer were mainly related to osmotic regulators (taurine and scyllo- and myo-inositol), citrate, and amino acids supplying the tricarboxylic acid cycle[54]. Importantly, FA was observed to possess metabolic features of benign thyroid lesions, while simultaneously exhibited part of a metabolic profile associated

with TC, thus indicating an intermediate nature of FA being a potential individual stage of thyroid cancer development[54].

In recent years, IMS has arisen as a promising technique to discover potential metabolic biomarkers as demonstrated in several studies[55,56]. IMS analysis of seven cases of TPC by comparison of thyroid cancer sections with and adjacent normal tissue was conducted, focusing on the distribution of phospholipids[55]. It was observed that phosphatidylcholine (16:0/18:1) and (16:0/18:2) and sphingomyelin (d18:0/16:1) were significantly higher in thyroid papillary cancer than in normal thyroid tissue[55]. More recently, 124 thyroid carcinoma patients (including PTC and FTC) were compared to 122 normal and 43 benign thyroid cases focusing on the serum levels of phosphatidylcholines, phosphatidic acids, and sphingomyelins using IMS analysis coupled with matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance mass spectrometry[56]. The potential biomarkers detected in serum showed potential diagnostic power to differentiate between cancer patients and healthy individuals as well as between patients with malignant and benign thyroid lesions with high sensitivity and specificity[56].

All these observations confirm that metabolomics has the potential to expand our knowledge on molecular factors involved in TC becoming an emerging research approach in this disease. Furthermore, metabolomics studies may improve the basic knowledge on cancer-related processes and on novel biomarkers, implementing its diagnosis and classification.

Conclusion

Thyroid carcinoma is the most common endocrine system malignancy with rising incidence worldwide. As in other cancers, there is a lack of reliable and specific markers for the

detection of the disease and for that reason investigations in thyroid pathology are mainly aimed at the development of new tools for better diagnosis and stratification. Proteomic and metabolomic analysis of TC has confirmed changes in several known biomarkers, identified several potential novel biomarkers, and provided insights into global pathophysiologic changes in TC. Moreover, with the advent of novel high-resolution approaches, protein isoform differences and post-translational modifications not accessible by genomic or older proteomic approaches have been elucidated. These findings could be the starting point for further investigations aimed at translating these results into clinical practice with carefully designed and controlled prospective studies. The coming of age of proteomics and metabolomics makes available a formidable technological resource to further expand our knowledge of the complexities of human disease. The standardization, analysis and comprehensive collation of the "data-heavy" outputs of these sciences are indeed challenging. It is important to remark that proteomics, metabolomics and other Omic sciences have the singular advantage of being complimentary for cross validation, and together could potentially enable novel perspectives of biological systems and the perturbations underlying disease processes.

Declaration of interests

The authors report no declarations of interest.

Acknowledgements

Dr. Orenes-Piñero is supported by a postdoctoral contract from the Instituto Murciano de Investigaciones Biosanitarias Virgen de la Arrixaca (IMIB-Arrixaca, Murcia, Spain).

References

1. Navas-Carrillo D, Ríos A, Rodríguez JM, et al. Familial nonmedullary thyroid cancer: screening, clinical, molecular and genetic findings. *BBA-Rev Cancer* 2014;1846:468-76.

2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. CA Cancer J Clin. 2017;67:7-30.

3. Bonora E, Tallini G, Romeo G. Genetic Predisposition to Familial Nonmedullary Thyroid Cancer: An Update of Molecular Findings and State-of-the-Art Studies. *J Oncol* 2010;2010:385206.

4. El-Naggar AK, Chan JKC, Grandis JR, et al, editors. WHO/IARC Classification of Tumours. 4th ed. Vol 9, WHO/IARC Classification of Head and Neck Tumours. IARC Publications; 2017.

5. Paschke R, Cantara S, Crescenzi A, et al. European Thyroid Association Guidelines regarding Thyroid Nodule Molecular Fine-Needle Aspiration Cytology Diagnostics. *Eur Thyroid J*. 2017;6:115-29.

6. Martínez-Aguilar J, Clifton-Bligh R, Molloy MP. Proteomics of thyroid tumours provides new insights into their molecular composition and changes associated with malignancy. *Sci Rep* 2016;6:23660.

7. Kenyon GL, DeMarini DM, Fuchs E, et al. Defining the mandate of proteomics in the post-genomics era: workshop report. *Mol Cell Proteomics* 2002;1:763-80.

8. Orenes-Piñero E, Cortón M, González-Peramato P, et al. Searching urinary tumor markers for bladder cancer using a two-dimensional differential gel electrophoresis (2D-DIGE) approach. *J Proteome Res* 2007;6:4440-8.

9. Navas-Carrillo D, Marín F, Valdés M, et al. Deciphering acute coronary syndrome biomarkers: High-resolution proteomics in platelets, thrombi and microparticles. *Crit Rev Clin Lab Sci*. 2017;54:49-58.

10. Keshishian H, Burgess MW, Gillette MA, et al. Multiplexed, quantitative workflow for sensitive biomarker discovery in plasma yields novel candidates for early myocardial injury. *Mol Cell Proteomics* 2015;14:2375-93.

11. Farrokhi-Yekta R, Rezaie-Tavirani M, Arefi-Oskouie A, et al. The metabolomics and lipidomics window into thyroid cancer research. *Biomarkers* 2016;15:1-9.

12. Wojakowska A, Chekan M, Widlak P, et al. Application of metabolomics in thyroid cancer research. *Int J Endocrinol* 2015; 2015:258763.

13. Duarte IF, Gil AM. Metabolic signatures of cancer unveiled by NMR spectroscopy of human biofluids. *Prog Nucl Magn Reson Spectrosc* 2012;62:51-74.

14. Brown LM, Helmke SM, Hunsucker SW, et al. Quantitative and qualitative differences in protein expression between papillary thyroid carcinoma and normal thyroid tissue. *Mol Carcinog* 2006;45:613-26.

15. Netea-Maier RT, Hunsucker SW, Hoevenaars BM, et al. Discovery and validation of protein abundance differences between follicular thyroid neoplasms. *Cancer Res* 2008;68:1572-80.

16. Mato E, Barceló-Batllori S, Orera I, et al. The proteomic 2D-DIGE approach reveals the protein voltage-dependent anion channel 2 as a potential therapeutic target in epithelial thyroid tumours. *Mol Cell Endocrinol* 2015;404:37-45.

17. Paricharttanakul NM, Saharat K, Chokchaichamnankit D, et al. Unveiling a novel biomarker panel for diagnosis and classification of well-differentiated thyroid carcinomas. *Oncol Rep* 2016;35:2286-96.

18. Ross PL, Huang YN, Marchese JN, et al. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 2004;3:1154–69.

19. Ruppen I, Grau L, Orenes-Piñero E, et al. Differential protein expression profiling by iTRAQ-two-dimensional LC-MS/MS of human bladder cancer EJ138 cells transfected with the metastasis suppressor KiSS-1 gene. *Mol Cell Proteomics* 2010;9:2276-91.

20. Mainini V, Lalowski M, Gotsopoulos A, et al. MALDI-imaging mass spectrometry on tissues. *Methods Mol Biol* 2015;1243:139-64.

21 Min KW, Bang JY, Kim KP, et al. Imaging mass spectrometry in papillary thyroid carcinoma for the identification and validation of biomarker proteins, *J. Korean Med. Sci* 2014;29:934-40.

22. Mosele N, Smith A, Galli M, et al. MALDI-MSI Analysis of Cytological Smears: The Study of Thyroid Cancer. *Methods Mol Biol.* 2017;1618:37-47.

23. Mainini V, Pagni F, Ferrario F, et al. MALDI imaging mass spectrometry in glomerulonephritis: feasibility study. *Histopathology* 2014;64:901-6.

24. Meding S, Nitsche U, Balluff B, et al. Tumor classification of six common cancer types based on proteomic profiling by MALDI imaging. *J. Proteome Res* 2012;11:1996-2003.

25. Lei Z, Huhman DV, Sumner LW. Mass spectrometry strategies in metabolomics. *J Biol Chem* 2011;286: 25435-42.

26. Serkova NJ, Spratlin JL, Eckhardt SG. NMR-based metabolomics: translational application and treatment of cancer. *Curr Opin Mol Ther* 2007;9:572-85.

27. Liu X, Locasale JW. Metabolomics: A Primer. *Trends Biochem Sci* 2017;42:274-84.

28. Kouremenos KA, Johansson M, Marriott PJ. Advances in gas chromatographic methods for the identification of biomarkers in cancer. *J Cancer* 2012;3:404-20.

29. Link H, Fuhrer T, Gerosa L, et al. Real-time metabolome profiling of the metabolic switch between starvation and growth. *Nat. Methods* 2015;12:1091-7.

30. Contrepois K, Jiang L, Snyder M. Optimized analytical procedures for the untargeted metabolomic profiling of human urine and plasma by combining hydrophilic interaction (hilic) and reverse-phase liquid chromatography (RPLC)–Mass spectrometry. *Mol Cell Proteom* 2015;14:1684-95.

31. Rosai J. Immunohistochemical markers of thyroid tumors: significance and diagnostic applications. *Tumori* 2003;89:517-9.

32. Dunn AD, Crutchfield HE, Dunn JT. Thyroglobulin processing by thyroidal proteases. Major sites of cleavage by cathepsins B, D, and L. *J Biol Chem* 1991;266:20198-204.

33. Gondi CS, Lakka SS, Yanamandra N, et al. Adenovirus-mediated expression of antisense urokinase plasminogen activator receptor and antisense cathepsin B inhibits tumor growth, invasion, and angiogenesis in gliomas. *Cancer Res* 2004;64:4069-77.

34. Zafon C, Obiols G, Castellví J, et al. nm23-H1 immunoreactivity as a prognostic factor in differentiated thyroid carcinoma. *J Clin Endocrinol Metab* 2001;86:3975-80.

35. Maldonado EN, Sheldon KL, DeHart DN, et al. Voltage-dependent anion channels modulate mitochondrial metabolism in cancer cells: regulation by free tubulin and erastin. *J Biol Chem* 2013;288:11920-9.

36. Lazarou M, Stojanovski D, Frazier AE, et al. Inhibition of bak activation by VDAC2 is dependent on the bak transmembrane anchor. *J Biol Chem* 2010;285:36876-83.

37. Cheng EHY, Sheiko TV, Fisher JK, et al. VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science* 2003;301:513-7.

38. Dinets A, Pernemalm M, Kjellin H, et al. Differential protein expression profiles of cyst fluid from papillary thyroid carcinoma and benign thyroid lesions. *PLoS One* 2015;10:e0126472.

39. de Matos LL, Del Giglio AB, Matsubayashi CO, et al. Expression of CK-19, galectin-3 and HBME-1 in the differentiation of thyroid lesions: systematic review and diagnostic meta-analysis. *Diagn Pathol* 2012;7:97.

40. Sofeu-Feugaing DD, Gotte M, Viola M. More than matrix: the multifaceted role of decorin in cancer. *Eur J Cell Biol* 2013; 92:1-11.

41. Clementz AG, Harris A. Collagen XV: exploring its structure and role within the tumor microenvironment. *Mol Cancer Res* 2013;11:1481-6.

42. Orend G, Chiquet-Ehrismann R. Tenascin-C induced signaling in cancer. *Cancer Lett* 2006;244:143-63.

43. Ko KH, Han NY, Kwon CI, et al. Recent advances in molecular imaging of premalignant gastrointestinal lesions and future application for early detection of Barrett esophagus. *Clin Endosc* 2014;47:7-14.

44. Meding S, Nitsche U, Balluff B, et al. Tumor classification of six common cancer types based on proteomic profiling by MALDI imaging. *J Proteome Res* 2012;11:1996-2003.

45. Nipp M, Elsner M, Balluff B, et al. S100-A10, thioredoxin, and S100-A6 as biomarkers of papillary thyroid carcinoma with lymph node metastasis identified by MALDI imaging. *J Mol Med (Berl)* 2012;90:163-74.

46. Galli M, Pagni F, De Sio G, et al. Proteomic profiles of thyroid tumors by mass spectrometry-imaging on tissue microarrays. *Biochim Biophys Acta.* 2017;1865:817-27.

47. Pagni F, De Sio G, Garancini M, et al. Proteomics in thyroid cytopathology: Relevance of MALDI-imaging in distinguishing malignant from benign lesions. *Proteomics* 2016;16:1775-84.

48. Park WS, Chung KW, Young MS, et al. Differential protein expression of lymph node metastases of papillary thyroid carcinoma harboring the BRAF mutation. *Anticancer Res* 2013;33:4357-64.

49. Carpenter B, MacKay C, Alnabulsi A, et al. The roles of heterogeneous nuclear ribonucleoproteins in tumour development and progression. *Biochim Biophys Acta* 2006;1765:85-100.

50. Russell P, Lean CL, Delbridge L, et al. Proton magnetic resonance and human thyroid neoplasia. I: Discrimination between benign and malignant neoplasms. *Am J Med* 1994;96:383-8.

51. Mackinnon WB, Delbridge L, Russell P, et al. Two-dimensional proton magnetic resonance spectroscopy for tissue characterization of thyroid neoplasms. *World J Surg* 1996;20:841-7.

52. Yoshioka Y, Sasaki J, Yamamoto M, et al. Quantitation by (1)H-NMR of dolichol, cholesterol and choline-containing lipids in extracts of normal and phathological thyroid tissue. *NMR Biomed* 2000;13:377-83.

53. Miccoli P, Torregrossa L, Shintu L, et al. Metabolomics approach to thyroid nodules: a high-resolution magic-angle spinning nuclear magnetic resonance-based study. *Surgery* 2012;152:1118-24.

54. Deja S, Dawiskiba T, Balcerzak W, et al. Follicular adenomas exhibit a unique metabolic profile. ¹H NMR studies of thyroid lesions. *PLoS One* 2013;8:e84637.

55. Ishikawa S, Tateya I, Hayasaka T, et al. Increased expression of phosphatidylcholine (16:0/18:1) and (16:0/18:2) in thyroid papillary cancer. *PLoS One* 2012;7:e48873.

56. Guo S, Qiu L, Wang Y, et al. Tissue imaging and serum lipidomic profiling for screening potential biomarkers of thyroid tumors by matrix-assisted laser desorption/ionization-Fourier transform ion cyclotron resonance mass spectrometry. *Anal Bioanal Chem* 2014;406:4357-70.

Tables

Table 1: Proteomic analysis searching for novel potential biomarkers in TC

Authors	Sample used	Proteomic Approach	Main Findings	Reference
Brown et al, 2006	5 PTC samples matched with normal thyroid tissue from the same patients	2D-DIGE	S100A6, peroxiredoxin 2, and HSP 70 were identified. S100A6 was validated using IHC, showing a sensitivity and specificity of 85% and 69%, respectively, in distinguishing benign from malignant lesions.	(14)
Netea-Maier et al, 2008	Protein extracts from 5 patients with FTC *vs* 6 patients with BFTA	2D-DIGE	HSP gp96, PDI A3, and calreticulin were identified differentially expressed. The combination of all these markers showed a positive predictive value of 75% and a negative predictive value of 68%.	(15)
Mato et al, 2015	Frozen tissues of NTT (n=15), PTC (n=5), FTC (n=4), ATC (n=3), and ADE (n=4)	2D-DIGE	AIFM1, HIBCH, peroxiredoxin 3, peroxiredoxin 5, UQCRC2 and VDAC2 were identified. A double-validation confirmed the over-expression of VDAC2 and its role in resistance to apoptosis previously observed.	(16)
Parichartanakul et al, 2016	5 PTC and 5 FTC tissues, with their adjacent NTT from Thai patients.	2D-DIGE	Enolase 1, TPI, cathepsin D, annexin A2, cofilin 1, PCNA, copine 1 and HSP27 distinguished PTC from NTT; all except Annexin A2 can discriminate FTC from normal tissues. Annexin A2, cofilin 1, PCNA and HSP27 distinguished PTC *vs* FTC.	(17)
Dinets et al, 2015	20 cPTCs and 56 benign cystic thyroid lesions	iTRAQ	CK-19, S100A13, annexin A3, CMBL were differentially expressed and further validated. CK-19 levels in cyst fluid can complement FNAB for discrimination of cPTC from benign thyroid cysts.	(38)
Martínez-Aguilar et al, 2016	Fresh frozen tissues from NTT, FA, minimally invasive FTC and PTC	iTRAQ	FA and FTC, showed marked reduction (>10-fold) of decorin. TGFBI could correctly differentiate most FTC samples from the FA group with a high fold change.	(6)
Nipp et al, 2012	29 PTC patients	MALDI-IMS	Thioredoxin, S100-A10 and S100-A6 were found highly associated with lymph node metastasis in PTC ($p<0.005$).	(45)
Galli et al, 2016	44 FFPE samples including HP, FA and PTC	MALDI-IMS	FINC, ACTB1, LMNA, HSP7C and KAD1 were upregulated specifically in the neoplastic tissues, thus representing potential markers of malignancy.	(46)
Pagni et al, 2016	26 FNAB; 13 benign thyroid nodules and 13PTC	MALDI-IMS	MALDI-IMS was able to correctly assign, in blind, the specimens to a malignant or benign class, as later confirmed by the morphological classification.	(47)

Abbreviations: 2D-DIGE: Two-dimensional differential in gel electrophoresis; HSP 70: Heat-shock protein-70; IHC: Immunohistochemistry; FTC: Folicular thyroid cancer; BFTA: benign follicular thyroid adenoma; PDI A3: protein disulfide isomerase A3; NTT: Normal thyroid tissue; PTA: Papillary thyroid cancer; ATC: Anaplastic thyroid cancer; ADE: Adenoma; AIFM1: Apoptosis inducing factor mitochondrion-associated 1; HIBCH: 3-hydroxyisobutyryl-CoA hydrolase mitochondrial; UQCRC2: Ubiquinolcytochrome c reductase core protein II; VDAC2: Voltage-dependent anion channel 2; TPI: triose phosphate isomerase; PCNA: Proliferating cell nuclear antigen; cPTC: Cystic variant of PTC; iTRAQ: Isobaric tag for relative and absolute quantification; CK-19: Cytokeratin-19; CMBL: carboxymethylenebutenolidase homolog; FNAB: Fine-needle aspiration biopsy; FA: Follicular adenoma; TGFBI: TGFβ-induced protein ig-h3; FFPE: Formalin-fixed paraffin embedded; HP: Hyperplastic nodule; FINC: fibronectin; ACTB1: Cytoplasmic actin 1; LMNA: Prelamin A/C; KAD1: Adenylate Cyclase Isoenzyme 1.

Figures

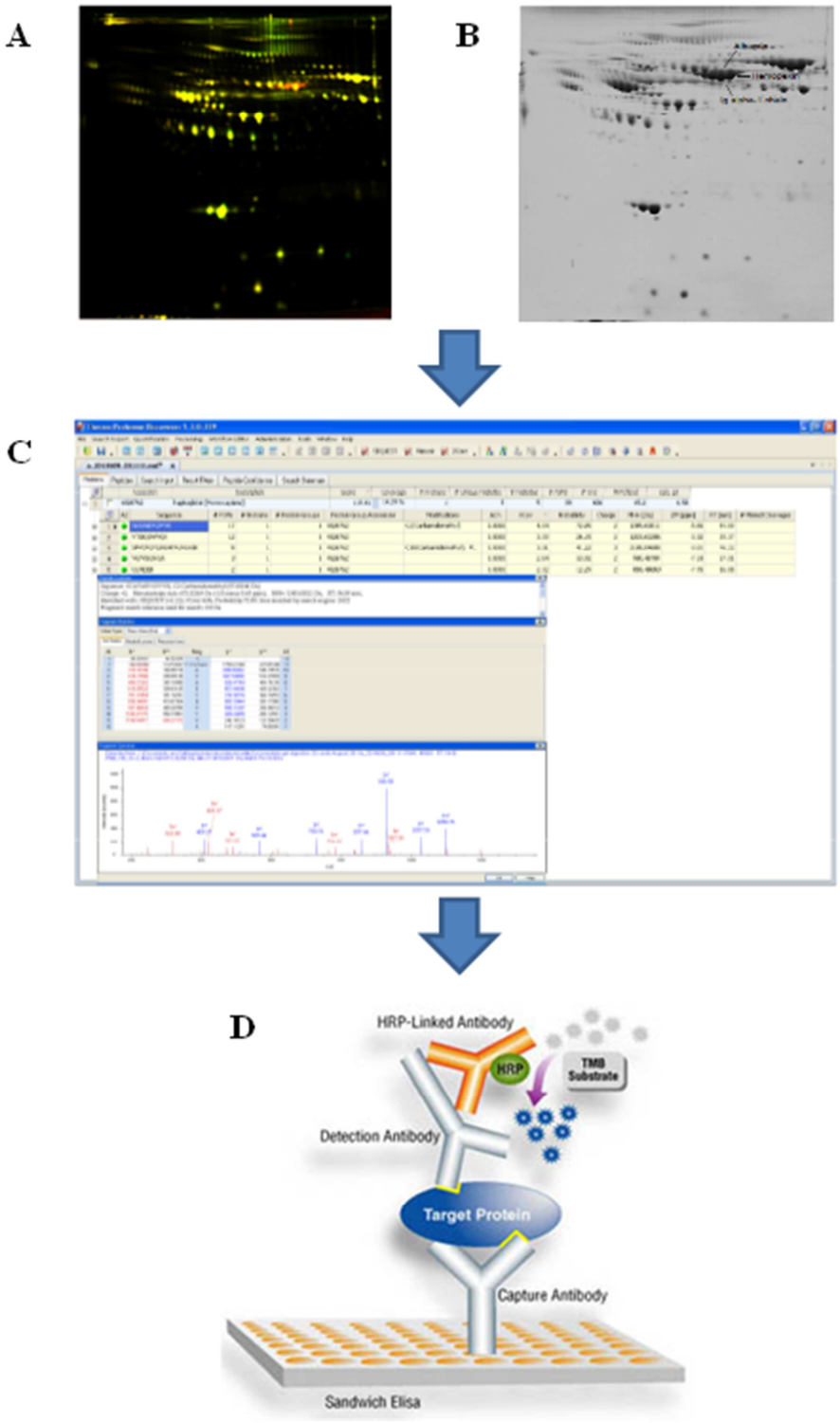

Figure 1. Experimental design of 2D-DIGE technique. **A)** 2D-DIGE analyses reveal proteins differentially expressed in a significant manner. **B)** Preparative new gels with higher protein concentration can be prepared for picking up selected spots. **C)** Proteins of interest were identified using mass spectrometry. **D)** ELISA serves to validate the diagnostic properties of identified proteins on independent series of specimens.

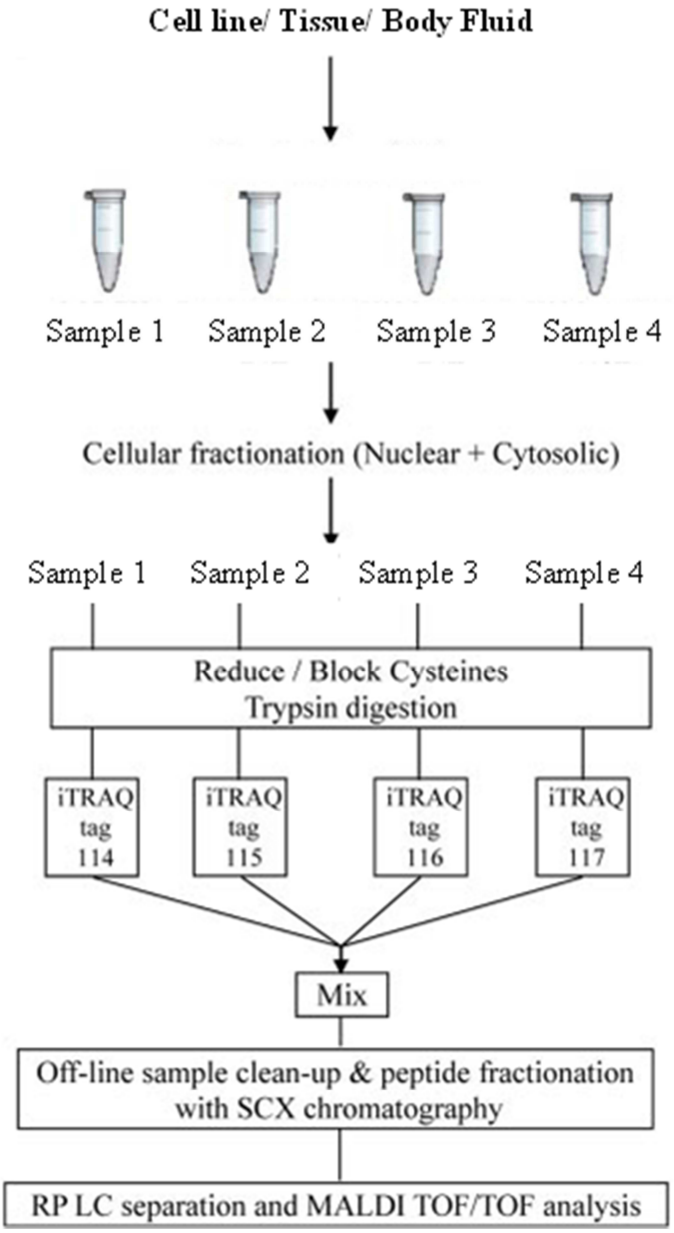

Figure 2. Schematic diagram showing the design work flow used for the multiplexed iTRAQ-based experiments.

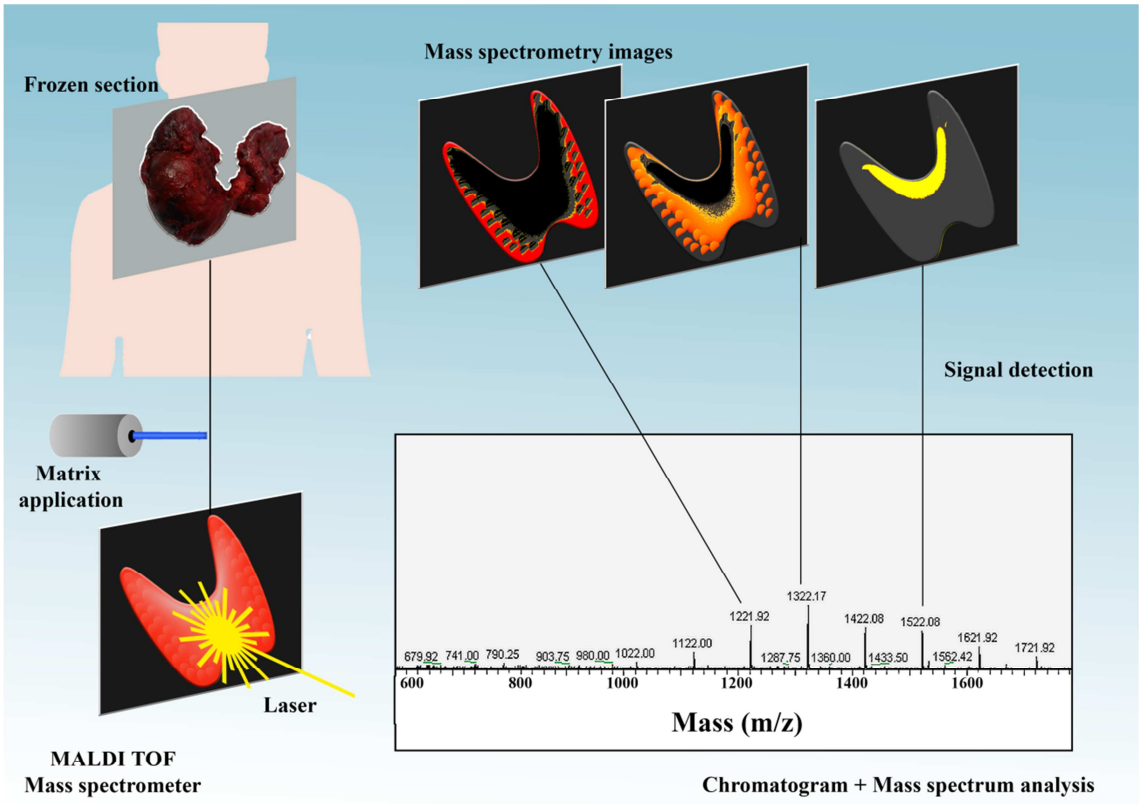

Figure 3. The principle of Imaging Mass Spectrometry.