PROTEOMIC ANALYSIS OF PLASMA MOLECULAR MARKERS AS PREDICTORS OF DIFFERENTIATED THYROID CANCER

A.E. BACIU1, 2, E. UYY3, V. I. SUICA3, R.M. BOTEANU3, A.I. POPESCU1, C. GIULEA4, 5, D. MANDA2, C. BADIU2, 5, F. ANTOHE3

1 University of Bucharest, Faculty of Physics, P.O. Box MG-11, Magurele, Romania
2 National Institute of Endocrinology “C. I. Parhon”, Bucharest, Romania
3 “Nicolae Simionescu” Institute of Cellular Biology and Pathology, Bucharest, Romania
4 Elias University Clinical Hospital, Bucharest, Romania
5 “Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania
E-mail: feliciaantohe@icbp.ro

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Abstract. The differentiated thyroid cancer is the most frequent endocrine cancer with unpredicted biological evolution. This study aimed to identify potential biomarkers from plasma microvesicles, for early detection, diagnosis and prognosis of cancer versus benign follicular adenoma thyroid lesions. Comparative proteomic analysis between tissue and plasma microvesicles identified eight proteins whose abundances were altered: Filamin-A, Ras-related C3 botulinum toxin substrate 1, integrin beta-1, moesin, CD44 antigen, ezrin, vitronectin, and cell division control protein 42 homolog. These proteins in association with the glycosaminoglycans, hyaluronan and heparan sulfate proteoglycans revealed significant alteration levels that may demonstrate participation in tumourigenesis and metastasis.

Key words: differentiated thyroid cancer, microvesicles, plasma proteins, mass spectrometry, molecular markers.

1. INTRODUCTION

In recent years, an increased incidence of thyroid tumours was recorded worldwide, including Romania [1–5]. Thyroid cancer is a common malignancy of the endocrine organs and occurs in the thyroid nodules [2]. Most common thyroid tumours are benign follicular adenomas (A) and differentiated thyroid cancer (DTC), the latter presenting an unexpected biological evolution such as aggressive cell proliferation [1, 3]. DTC includes papillary carcinoma and follicular carcinoma with predominant incidence in women [6]. Approximately 5% of diagnosed patients could develop metastases and have a high death risk [7]. Currently, the treatment is based on the diagnosis risk group and may include surgery, radioactive iodine therapy and levothyroxine substitution for thyroid-stimulating hormone (TSH) suppression [8, 9]. Radioactive iodine therapy often leads to positive
response and could result in reduced long-term morbidity, re-occurrence and possible mortality [10]. The follow-up includes periodic measurements of serum thyroglobulin (molecular marker of thyroid cancer evolution), thyroid stimulating hormone, neck ultrasound investigation and scintigraphy scan of the entire body for metastases identification [7-9]. Recent findings have identified that the thyroid cancer releases, in the blood, microvesicles (MVs) containing genetic and cellular information [11, 12]. Mapping their biochemical composition could lead to identification of specific biomarkers with diagnostic and prognostic value that may help in the design of a personalized treatment in order to improve the patients’ health [12-16]. The interest in protein detection from plasma isolated MVs has increased in recent decades due to the identification of biomarkers as predictors of unfavorable health evolution [12]. The plasma circulating MVs are phospholipid vesicles with a diameter between 100 and 1,000 nm, present in both normal and pathological states, playing a role in inter-cellular communication [17]. Published data showed that cancer can cause abnormal high concentrations of MVs in the blood with different biochemical compositions from patient to patient [17, 18]. MVs derived from tumour cells can induce angiogenesis during tumour growth and can contribute to the initiation of metastases [17–19]. The aim of the present study was to identify the potential molecular tumoural predictors from plasma MVs, using high performance mass spectrometry, which could facilitate the early detection and long term monitoring of thyroid neoplasia.

2. MATERIALS AND METHODS

2.1. TISSUE AND BLOOD COLLECTION AND PREPARATION

This study was approved by the Ethics Committee of the National Institute of Endocrinology “C. I. Parhon”, Bucharest. Biological samples were collected from 20 patients with differentiated thyroid carcinoma (DTC) and 20 with follicular adenoma (A), who underwent surgery in the National Institute of Endocrinology “C. I. Parhon” and Elias University Clinical Hospital, Bucharest, Romania. 10 volunteers were considered as control subjects (C), as previously described [20]. Blood samples (~ 3.5 mL) were collected prior to surgery (DTC_before; A_before) and one month after surgery (DTC_after; A_after). Tumoural (T) and non-tumoural (NT) adjacent tissue samples (100-200) mg were stored in phosphate buffered saline, at -80°C. Ethylenediaminetetraacetic acid (EDTA) coated tubes (K3E, Kabe Labortechnik) were used for plasma separation. MVs from plasma were isolated after a series of successive centrifugations [17]. Tissue fragments (30 mg) were homogenized in 0.3 mL buffer containing 8 M urea, 1% sodium deoxycholate (DOC) and 100 mM Tris-HCl (pH 7.5) with the use of a rotor-stator mechanical homogenizer. Protein quantification was performed using The Precision Red
Advanced Protein Assay Reagent 2 (Cytoskeleton, Denver, USA). The protein fraction extracted from the supernatant was analyzed by liquid chromatography-mass spectrometry (LC-MS).

2.2. LIQUID CHROMATOGRAPHY-MASS SPECTROMETRIC ANALYSIS

Liquid nano-chromatography tandem mass spectrometry (LC-MS/MS) experiments were performed using the EASY-nLC II nano system coupled to the LTQ-Velos Pro Orbitrap mass spectrometer (Thermo Scientific, CA, USA). C18 solid phase extraction (SPE) columns were purchased from Waters (MA, USA). Urea, DOC, trizma hydrochloride (Tris-HCl), DL-dithiothreitol (DTT), iodoacetamide (IAA), N-acetyl-L-cysteine (NAC), ammonium bicarbonate and all of the solvents were provided by Sigma-Aldrich (Missouri, USA). Trypsin Gold was acquired from Promega (Wisconsin, USA). The samples were purified by acetone precipitation (1 h, at –20 °C) followed by 20 min centrifugation (20,000×g). The protein pellet was suspended in a reducing buffer containing 8 M urea, 0.1 M Tris-HCl (pH 8.8), 0.1 mM EDTA and 20 mM DTT. Alkylation of the proteins was conducted using 80 mM IAA in 0.1 M Tris-HCl and 0.1 mM EDTA buffer, for 90 min, in the dark, under agitation, while the IAA excess was quenched using 80 mM NAC in 0.1 M Tris-HCl and 0.1 mM EDTA buffer, for 30 min. Proteolysis was performed overnight, at 37 °C, using a 1:20 enzyme to substrate ratio. The peptides were purified by SPE and concentrated using the Concentrator plus system (Eppendorf, Hamburg, Germany), prior to LC-MS/MS analysis. Peptides were eluted in triplicate using acetonitrile with 0.1% (v/v) formic acid over water with 0.1% (v/v) formic acid by a 2–35% separation gradient at a flow rate of 300 nL/min. The MS was operated in a top 12 data dependent configuration at 60k resolving power. Protein identification and label free relative quantification were performed using Proteome Discoverer SIEVE, 2.1 (Thermo Scientific) and Mascot 2.4.1 (Matrix Science, London, UK) search engine. SIEVE aligns mass spectra and compares precursor ion intensities [21]. Protein Center software was used for annotation and projection of the quantitative data onto Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathways.

3. RESULTS AND DISCUSSIONS

3.1. QUALITATIVE BIOINFORMATICS ANALYSIS OF MICROVESICLE PROTEINS

A total of 1,218 proteins from pre-surgery plasma MVs were identified in all three groups: 874 proteins in DTC, 921 proteins in A, and 552 proteins in C group.
253 of these proteins were specific only for group A, while 215 proteins were uniquely identified in DTC group and 66 in group C. 445 proteins were commonly attributed to all groups (Fig. 1).

The commonly identified MVs proteins in all three groups were matched with KEGG databases revealing several statistically significant over-represented (FDR P-value < 0.05; FDR: False Discovery Rate) signaling pathways. Among them, proteoglycans in cancer (KEEG 05205) inter-relation map proved to be particularly correlated with cancer pathology.

3.2. MICROVESICLE AND THYROID TISSUE LABEL FREE RELATIVE PROTEIN QUANTIFICATION

The tissue sample quantitative analysis comparing T with NT sets indicated ten statistically significant differentially abundant proteins, involved in proteoglycans in cancer KEGG 05205 pathways (Table 1).

Out of the ten proteins, nine presented a statistically significant up-regulation trend. These proteins, shown in Table 1, were: CD44 antigen (CD44), integrin beta-1 (ITGB1), cell division control protein 42 homolog (CDC42), Filamin-A (FLNA), Ras-related C3 botulinum toxin substrate 1 (RAC1), vitronectin (VTN), ezrin (EZR), moesin (MSN) and lumican (LUM). Remarkably, except lumican, the other eight proteins were found to be differentially expressed in plasma derived MVs isolated from A and DTC groups versus C group.

In differentiated thyroid carcinoma (DTC) there were 4 proteins with increased abundance in MVs after surgery (TGB1, FLNA, EZR and MSN). Interestingly, VTN abundance decreased after surgery, while in follicular adenoma (A) there were 3 proteins with increased abundance in MVs after surgery (ITGB1,
FLNA, EZR) and 3 proteins with decreased abundance (CD44, CDC42 and MSN) also after surgery. Table 2 summarizes the evolution of protein abundance in MVs after surgery according to diagnostic groups examined and related to the KEGG proteoglycans signaling pathways [22]. Previously published data demonstrated that the identified proteins were found to be involved in carcinogenesis together with glycosaminoglycans, hyaluronan (HA) and heparan sulfate proteoglycans (HSPGs) [23].

Table 1

<table>
<thead>
<tr>
<th>UniProt code</th>
<th>Description (Official symbol)</th>
<th>Thyroid tissue protein normalized abundance ratio (T/NT)</th>
<th>Thyroid tissue Median Mascot score</th>
<th>Microvesicles Median Mascot score</th>
</tr>
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<tr>
<td>P16070</td>
<td>CD44 antigen (CD44)</td>
<td>2.84±0.43**</td>
<td>1.88±0.34**</td>
<td>81</td>
</tr>
<tr>
<td>P51884</td>
<td>Lumican (LUM)</td>
<td>0.99±0.09***</td>
<td>0.54±0.052***</td>
<td>1271</td>
</tr>
<tr>
<td>P05556</td>
<td>Integrin beta-1 (ITGB1)</td>
<td>2.36±0.34**</td>
<td>1.75±0.236**</td>
<td>159</td>
</tr>
<tr>
<td>P60953</td>
<td>Cell division control protein 42 homolog (CDC42)</td>
<td>2.17±0.28***</td>
<td>3.58±0.523***</td>
<td>211</td>
</tr>
<tr>
<td>P21333</td>
<td>Filamin-A (FLNA)</td>
<td>1.59±0.15***</td>
<td>1.31±0.079**</td>
<td>1045</td>
</tr>
<tr>
<td>P63000</td>
<td>Ras-related C3 botulinum toxin substrate 1 (RAC1)</td>
<td>2.16±0.43**</td>
<td>1.74±0.459**</td>
<td>124</td>
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<tr>
<td>P56706</td>
<td>Basement membrane-specific heparan sulfate proteoglycan core protein (HSPG2)</td>
<td>1.23±0.05***</td>
<td>1.37±0.083***</td>
<td>4324</td>
</tr>
<tr>
<td>P04004</td>
<td>Vitronectin (VTN)</td>
<td>3.6±0.33***</td>
<td>1.83±0.202*</td>
<td>704</td>
</tr>
<tr>
<td>P15311</td>
<td>Ezrin (EZR)</td>
<td>2.48±0.38***</td>
<td>2.96±0.607*</td>
<td>634</td>
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<tr>
<td>P26038</td>
<td>Moesin (MSN)</td>
<td>1.98±0.19***</td>
<td>2.29±0.306**</td>
<td>1029</td>
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</table>

Proteoglycans are proteins that are heavily glycosylated, being active factors in the tumour microenvironment that contribute to the progression of various types of cancer by enhancing the proliferation, angiogenesis and metastasis processes [24–26]. Hyaluronan is a member of hyaluronan synthases family and is the main constituent of proteoglycans present in extra-cellular matrix [27]. HA interacts with CD44 receptor and contributes to the growth and survival of tumour cells. Heparan sulfate proteoglycans (HSPGs) are binding glycoproteins components of the extra-cellular matrix, which promote cell migration. Along with integrins and other cell
adhesion receptors; these glycoproteins facilitate cell-cell interaction and cell motility [28].

Fig. 2 – Mass spectrometric proteomic abundance analysis of the eight proteins in plasma MVs, harvested prior to surgery (DTC_before and A_before) and after the surgery (DTC_after and A_after) versus control groups. Graphs show comparative normalized relative abundance in arbitrary units of CD44 antigen (CD44), Integrin beta-1 (ITGB1), Cell division control protein 42 homolog (CDC42), Filamin-A (FLNA), Ras-related C3 botulinum toxin substrate 1 (RAC1), Vitronectin (VTN), Ezrin (EZR), Moesin (MSN). Data are represented as median ± standard deviation (SD).

<table>
<thead>
<tr>
<th>UniProt KB acc. code</th>
<th>Protein description</th>
<th>Official protein symbol</th>
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<th>A_after vs. A_before</th>
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<tr>
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<td>CD44 antigen</td>
<td>CD44</td>
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</tr>
<tr>
<td>P05556</td>
<td>Integrin beta-1</td>
<td>ITGB1</td>
<td>↗</td>
<td>↗</td>
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<tr>
<td>P60953</td>
<td>Cell division control protein 42 homolog</td>
<td>CDC42</td>
<td>↔</td>
<td>↙</td>
</tr>
<tr>
<td>P21333</td>
<td>Filamin-A</td>
<td>FLNA</td>
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<td>↗</td>
</tr>
<tr>
<td>P63000</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
<td>RAC1</td>
<td>↗</td>
<td>↔</td>
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<tr>
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<td>Vitronectin</td>
<td>VTN</td>
<td>↗</td>
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<tr>
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<td>Ezrin</td>
<td>EZR</td>
<td>↗</td>
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<tr>
<td>P26038</td>
<td>Moesin</td>
<td>MSN</td>
<td>↗</td>
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</tr>
</tbody>
</table>

CD44 has a role in cell migration, development, and progression of tumours [29]. It is expressed especially in papillary thyroid carcinoma in women under 45 years old, its level being lower in follicular thyroid carcinoma [30]. Different
groups showed that CD44 is expressed in both malignant and benign thyroid tissues [30–32], confirmed also by the present LC-MS study. In the metastatic gastric carcinoma, its serum concentration is correlated with the extent of tumour invasion and metastasis [33]. In our study, although the abundance of CD44 is not altered in the MVs isolated from the DTC patients, the A patients presented an up-regulation of its expression both in the tissue and the MVs harvested in the day of the surgery, whereas in the MVs isolated from the plasma collected 30 days after the surgery, its abundance decreased. In follicular adenoma, CD44 levels detected in MVs decreased after surgery (Fig. 2).

Integrins are important proteins in the process of tumour cell migration and growth, due to their involvement in the surviving signaling and regulation of cell adhesion [34]. The results of this study showed that integrin beta-1 (ITGB1) presents an up-regulation in the tissue samples in both DTC and A groups, together with an increased abundance in the MVs collected from the A before group. After the thyroid surgery, we observed a more than 20 fold increase in the abundance of ITGB1 in the DTC after and A after microvesicles. ITGB1 expression was found to be regulated by CDC42 at the transcriptional level in cancer cell lines [35]. CDC42 is a GTP-ase from Rho family expressed in various types of cancer, implicated in cell polarization, survival and migration. Also, it can promote the progression and metastasis in colon cancer [36]. Our results demonstrated that CDC42 abundance is increased in both thyroid tissue sample groups. In the MVs, isolated from the follicular adenoma patients before surgery (A before) the MS evaluated abundance of CDC42 was increased, while a significant down-regulation of the protein was observed in the follicular adenoma patients before surgery (A after) as shown in (Fig. 2).

FLNA is implicated in cell mobility and modulates DNA damage responses, with an important role in the inhibition of cancer metastasis [37]. It was found that in the FLNA deficient cancers, the radiation and chemotherapy were more effective, whereas in the tumours expressing FLNA the mentioned therapies were less sensitive with a higher risk of metastasis [37]. In the current LC-MS approach, the tissue level of FLNA was higher in both types of tumours than their corresponding control samples. The protein abundance of FLNA was significantly decreased in the DTC before and A before, while the reversed situation was observed for DTC after and A after patients (Fig. 2).

RAC1 is the GTPase from Ras superfamily involved in many cellular events including cellular growth control and cytoskeletal reorganization [38]. RAC1 is a protein that promotes the tumour progression of various processes through its signaling routes. Another important RAC1 characteristic is its direct involvement in pathways that regulate proliferation, inflammatory responses, G1 cell-cycle progression and the formation of the cell-cell contacts [39]. RAC1 was up-regulated in the thyroid tissue of both types of tumours. The MVs harvested from
the DTC\textsubscript{before}, those from DTC\textsubscript{after} and A\textsubscript{after} samples presented as well an up-regulation of RAC1 protein (Fig. 2).

VTN is a secreted plasma glycoprotein with a multifunctional role, involved in hemostasis, cell migration, and tumour malignant processes [40]. In the tissue samples of our study VTN protein levels were higher than the corresponding controls, whereas VTN abundance values were below the control ones, in all MVs samples considered in this study (Fig. 2).

EZR and MSN glycoproteins are part of the “Ezrin Radixin Moesin” family. They are involved in the mediation of actin-membrane linkages, the regulation of the dynamics and organization of the actin cytoskeleton regulating the Rho/Rac GTPase signaling cascade [41]. It is well known that EZR is overexpressed and implicated in various tumourigenic processes, such as invasion and metastasis in breast cancer, osteosarcoma, etc. Moreover, the up-regulation of EZR is usually correlated with a poor prognosis of subjects with cervical cancer, colorectal adenocarcinoma, and gastrointestinal cancers [42, 43]. In our study, EZR is up-regulated in both thyroid tissue sample groups as well as in the MVs isolated from the DTC\textsubscript{after} and A\textsubscript{after} patients. In the case of MSN, a similar trend was observed for the tissue samples and the MVs isolated from the DTC\textsubscript{after}, A\textsubscript{before} and A\textsubscript{after} patients (Fig. 2).

4. CONCLUSIONS

The high resolution mass spectrometry proteomic analysis clearly evidenced eight of the commonly identified proteins in all three patients groups (DTC, A and C) and differentially expressed in the plasma microvesicles. They were associated to hyaluronan and heparan sulfate proteoglycans, part of the “Proteoglycans in cancer” KEGG signaling pathway. The ITGB1, CDC42, VTN, and MSN pointed out a highly significant discrimination between benign follicular adenoma and malignant differentiated thyroid carcinoma.

The surgical removal of tumours induced the significant increase of ITGB1 and FLNA proteins’ abundances, in both types of thyroid tumours. In the case of DTC, after the surgery, RAC1, EZR and MSN presented a noteworthy overexpression. Our results demonstrated that some proteins may present a distinct and significant abundance values in the patients’ plasma as opposed to the control subjects. This may be a valuable indication of the pathological evolution of each group of thyroid lesions. However, these results should be taken with caution due to the reduced number of patients within the examined groups. Therefore further studies on larger patient cohorts should be performed in order to clinically validate these data.
Declaration of interest. A. E. Baciu, E. Uyy and V. I. Suica contributed equally to this work. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. Corresponding author: Felicia ANTOHE, PhD (felicia.antohe@icbp.ro).

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REFERENCES

32. V. De Falco, A. Tamburrino, S. Ventre, M. D. Castellone, M. Malek, S. N. Mani, M. Santoro, Cancer Res. 72, 1449-1458 (2012).