ARTICLE IN PRESS

Molecular and Cellular Endocrinology xxx (2017) 1–12

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Proteome profiles of different types of thyroid cancers

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A R T I C L E I N F O

Article history:
Received 5 May 2017
Received in revised form
20 October 2017
Accepted 23 November 2017
Available online xxx

Keywords:
Biomarkers
FFPE tissue
Mass spectrometry
Proteomics
Thyroid cancer

A B S T R A C T

Proteomics profiling of tissue specimens representative for major types of thyroid cancers: papillary (classical and follicular variant), follicular, anaplastic and medullary, as well as benign follicular adenoma, was performed using shotgun LC-MS/MS approaches. A combination of Orbitrap and MALDI-TOF approach allowed to identify protein products of 3700 unique genes and revealed large differences between medullary, anaplastic and epithelium-derived differentiated cancers (papillary and follicular). Proteins characteristic for medullary and anaplastic cancers included factors associated with neuroendocrine functions and factors typically associated with advanced malignancies, respectively. Proteomes of different types of epithelium-derived differentiated cancers and follicular adenoma were compared using multi-enzyme LC-MS/MS approach, which revealed products of 4800 unique genes. A comparable overall similarity of follicular cancers to both variants of papillary cancers was found. Moreover, follicular adenoma showed higher overall similarity to follicular cancer than to either variant of papillary cancer. Proteins discriminating differentiated thyroid neoplasms included factors associated with lipid and hormone metabolism, regulation of gene expression and maintenance of DNA structure. Importantly, proteome data matched several features of transcriptome and metabolome profiles of thyroid cancers contributing to systems biology of this malignancy.

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1. Introduction

Thyroid cancer is the most common endocrine cancer and contributes to 1–2% of all new malignancies diagnosed each year. Thyroid nodules are very common in the overall population - their reported prevalence varies from 3% to 68% (depending on the applied screening method and the evaluated population), while malignant tumors occur in less than 1% of such nodules. The majority of thyroid carcinomas originate from follicular epithelial cells and include well-differentiated papillary thyroid carcinomas (PTC; up to 80% of all thyroid malignancies) and follicular thyroid carcinomas (FTC; about 15% of thyroid malignancies). Moreover, anaplastic (undifferentiated) carcinoma (ATC; 1–2% of thyroid cancers), which is the most aggressive thyroid malignancy, also develops from epithelial cells. Additionally, medullary thyroid carcinoma (MTC) that is derived from parafollicular C-cells and has neuroendocrine features, comprises of 3–5% of thyroid cancers (Siegel et al., 2015; Pellegriti et al., 2013). Most of the thyroid tumors are diagnosed by pathomorphological assessment alone, and the resulting classification is the primary step in the assessment of prognosis and selection of a treatment (Hedinger et al., 1988; Salabè, 2001; DeLellis et al., 2004; Oertel, 2004; Haugen et al.,

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https://doi.org/10.1016/j.mce.2017.11.020
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Please cite this article in press as: Gawin, M., et al., Proteome profiles of different types of thyroid cancers, Molecular and Cellular Endocrinology (2017), https://doi.org/10.1016/j.mce.2017.11.020

2016). The majority of patients with thyroid cancer are diagnosed based on fine needle aspiration cytology (FNAC) of thyroid nodules (Sakorafas, 2010; Kakudo et al., 2014). Further diagnosis is performed based on histopathological intra- or post-operative examination of the resected thyroid tissue. However, in some cases cytological and histological patterns are ambiguous and proper classification is problematic (Faquin, 2008). In fact, thyroid tumors with follicular growth pattern include a broad range of lesions that are difficult to distinguish cytologically and can be challenging even in histologic specimens. These lesions include hyperplastic nodules, benign follicular thyroid adenomas (FA), follicular carcinomas, follicular variant of papillary thyroid carcinomas, and medullary thyroid carcinomas. On the other hand, PTC is the most overdiagnosed thyroid lesion, since it shares a number of cytological features with benign lesions (nodular hyperplasia, FA, Hashimoto's thyroiditis) as well as with other malignant lesions (e.g., Hürthle cell carcinoma, FTC, and MTC) (Cakir et al., 2002; DeLellis et al., 2004; Schlumberger, 2007; Ustun et al., 2014). Hence, for classification of thyroid tumors that exhibit unusual morphological patterns, additional tests are essential. However, molecular diagnostic tools have only a limited role in the routine diagnosis of thyroid cancer nowadays. Although in most cases the expression of calcitonin could reliably differentiate MTC from follicular epithelium-derived tumors (Bae et al., 2015), there is no specific biomarker used in the current clinical practice which would allow discrimination of other differentiated thyroid cancers. Though a number of candidate biomarkers were proposed to confirm the diagnosis of PTC, they were not widely tested in clinical practice yet (Panebianco et al., 2015). Similarly, several biomarker candidates were proposed for discrimination between follicular carcinoma and follicular adenoma, but none of them was positively validated, hence histologic evaluation remains a gold standard in a distinction between these lesions (Sobrinho-Simões et al., 2011).

Classification of thyroid cancers might be improved markedly if new biomarkers identified with the use of high-throughput „omics" approaches could support diagnosis based on histopathological patterns (Eszlinger and Paschke, 2010; Krause et al., 2009; Aragon Han et al., 2014; Pagni et al., 2015; Wojakowska et al., 2015a,b). The most numerous and advanced studies are based on mutation and gene expression profiling. This type of studies revealed several gene expression signatures, some of them associated with mutations in cancer driver genes, which enable distinguishing between different types of thyroid cancers or discrimination of malignant tumors from benign lesions (The Cancer Genome Atlas, 2014; Jarzab et al., 2005; Giordano et al., 2005; Eszlinger et al., 2007). A few proteomics studies also identified proteins that could discriminate between cancerous and normal thyroid tissue (Brown et al., 2006; Ban et al., 2012), malignant and benign follicular lesions (Netea-Maier et al., 2008; Uyy et al., 2016; Martínez-Aguilar et al., 2016), or papillary and follicular cancers (Sofiadis et al., 2012; Martínez-Aguilar et al., 2016). However, a systemic study that would allow comparing all types of thyroid cancers comprehensively (i.e., with high proteome coverage) is not yet available. Here we directly compared proteomes of five major types of thyroid malignancies: follicular and classical variant of PTC, FTC, ATC, and MTC, and the most frequent benign thyroid tumor: FA. In clinical practice formalin-fixed paraffin-embedded (FFPE) tissue specimens are used in verification/confirmation of thyroid cancer diagnosis, hence our proteomic study was performed using this archive material. As a result, we established proteomic signatures characteristic for different types of thyroid cancers, which expanded the basic knowledge on the biology of these malignancies and could be used in further validation studies aimed at identification of their specific biomarkers.

2. Materials and methods

2.1. Biological material

The research was performed for tissue material obtained from patients diagnosed with major types of thyroid tumors, namely: benign thyroid follicular adenoma (FA), classical and follicular variant of papillary thyroid carcinoma (PTC-CV and PTC-FV, respectively), follicular thyroid carcinoma (FTC), anaplastic thyroid carcinoma (ATC), medullary thyroid carcinoma (MTC). The material contained surgically resected tumor tissue with a surrounding margin of a normal tissue which was collected during a standard surgical treatment and secured for routine histopathologic analysis as a formalin-fixed paraffin-embedded (FFPE) specimen. All patients involved in the study underwent the surgery in the Maria Skłodowska-Curie Institute – Oncology Center in Gliwice without any previous treatment. The material was selected and described by an experienced pathologist form the local Tumor Pathology Department (selected specimens consisted of at least 50% of tumor cells, 80% on average). All procedures involving humans were conducted in compliance with the institutional and national regulations and the study was approved by the appropriate Bioethical Committee (permission no. KB/430-49/12). A detailed description of the material used in the study is presented in Supplementary File Table S1.

2.2. Preparation of samples prior to MS analysis

For each sample, four consecutive 10-μm-thick tissue sections were collected in a 1.5 mL tube and subjected to protein extraction via a modified procedure by Geoui et al. (Geoui et al., 2010). First, 500 μL of n-heptane was added to each tube followed by short vortex-mixing and incubation at room temperature for 1 h. Then 25 μL of methanol was added, mixed and centrifuged for 2 min. The supernatant was discarded and the pellet was mixed with 100 μL of a tissue lysis buffer (TLB; 0.1M Tris-HCl pH 8.0, 0.1M DDT, 4% SDS) and mixed thoroughly. Next, the samples were incubated in a thermoblock at 100 °C for 20min followed by incubation in a thermomixer at 80 °C for 2h with mixing (750 rpm). Every 10min the content of the tubes was mixed by inversion. After that, the tubes were cooled down at 4 °C for 1min and centrifuged for 15 min at 14000 g (4 °C). The supernatant was diluted with TLB and subjected to protein quantification with the use of tryptophan fluorescence method (Wiśniewski and Gaugaz, 2015). Each protein extract (75 μg) was subsequently subjected to purification in a ternary solvent mixture (methanol/chloroform/water, 200:50:150, v/v) and centrifuged at 9000 g for 2min. Then, the upper layer was discarded, the remaining layers were mixed with 150 μL methanol and centrifuged again (9000 g, 2min). Next, the supernatant was removed, the protein pellet was washed with 1 mL of acetone and centrifuged at 9000 g for 2min. The supernatant was removed and the pellet was left at 37 °C until dry. Finally, the pellet was mixed with 40 μL TLB and heated at 99 °C for 1h. Once cooled down to room temperature, the purified extracts were subjected to protein quantification with the use of tryptophan fluorescence method.

The purified protein extracts were subsequently subjected to a filter-aided sample preparation (FASP) procedure according to Wisniewski et al. (Wiśniewski et al., 2013). Briefly, a volume of each sample corresponding to 40 μg of total protein was loaded into a spin ultrafiltration unit (30 kDa cut-off) and proteins retained on the filtering membrane were purified from TLB lysis buffer by repeated washes with 8M urea in 0.1M Tris-HCl pH 8.5, followed by alkylation with iodoacetamide (0.05M). Then, digestion with endoproteases was performed: (i) with trypsin alone (enzyme to protein ratio1:100, w/w, 37 °C, 18h), or (ii) with endoproteinase

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