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Identification of biomarkers for diagnosis of thyroid cancer and exploration of their functional role in cancer development

Doctoral thesis

Departments of Molecular Biology

Faculty of biology

Author: Artūrs Ābols

Supervisor: Dr. biol Aija Linē

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Biology
Riga, Latvia

Chairman: Dr. biol. Kaspars Tārs
(University of Latvia, Riga, Latvia)

Opponents: Prof., Dr. med. Massimo Tonacchera
(University of Pisa, Pisa, Italy)

Prof., Dr. biol. Edvīns Miklaševičs
(Pauls Stradiņš Clinical University Hospital, Riga, Latvia)

Dr. med. Liene Ņikitina - Zaķe
(Latvian Biomedical research and study centre, Riga, Latvia)

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Abstract

The prevalence of palpable thyroid nodules ranges from 3 to 7% in the general population. The great majority of them are benign lesions, however 5 to 8 % of nodules prove to be malignant. Approximately 90-95% of thyroid cancers are well-differentiated papillary (PTC) or follicular (FTC) carcinomas, which are derived from thyroid follicular cells, ~ 3% are medullary carcinomas, formed from C cells and 2-7 % are low- differentiated or anaplastic carcinomas (ATC) that most likely develop by progression of PTC or FTC, are highly aggressive and have no effective treatment for now. Currently, the cytological examination of fine needle aspiration (FNA) biopsies is the standard technique for the pre-operative differential diagnosis of thyroid nodules. However, the results may be noninformative in ~20% of cases due to an inadequate sampling and the lack of highly specific, measurable cytological criteria, therefore ancillary biomarkers that could aid in these cases are clearly needed. Furthermore, the identification of serological biomarkers capable to discriminate between malignant and benign nodules would allow to develop a non-invasive assay for the detection of thyroid cancer and avoid problems associated with the sampling of biopsies. Therefore the aims of the doctoral thesis were the following:

(i) To identify and validate new gene expression biomarkers in thyroid cancer tissues, which could be used for the development of pre - operative diagnostic test for thyroid cancer;
(ii) To elucidate the functional significance of TFF3, one of the best tissue biomarkers, in the development of thyroid cancer;
(iii) To explore the repertoire of cancer-associated autoantibodies in patients with thyroid cancer in order to identify autoantibody-based biomarkers for the development of non-invasive biomarker assays.

The first aim resulted in the generation of a complex model based on 6 gene expression signature (*LGALS3, BIRC5, CCND1, CITED1; MET* and *TFF3*) that outperformed the single marker and two- gene biomarker models (AUC=0.895; Sn - 70.5%; Sp - 93.4% with p value <0,0001) and clearly warrants further studies in FNAB.

The TFF3 functional study showed that in thyroid cancer, on the contrary to many other epithelial cancers,TFF3 mRNA and protein expression is downregulated. Forced expression of TFF3 in anaplastic thyroid cancer cell line 8305C resulted in decreased cell proliferation, clonal spheroid formation and entry into the S phase. Furthermore, it induced acquisition of epithelial-like cell morphology and expression of the differentiation markers of thyroid follicular cells and transcription factors implicated in the thyroid morphogenesis and function. Based on these data and previously published results in other cancer types, we propose that TFF3 can act as a tumor suppressor or oncogene in the cell context dependent manner.

Exploration of the repertoire of cancer associated autoantibodies in patients with thyroid cancer against 65 tumor associated antigens resulted in the detection of anti-GAGE1 autoantibodies in 5% of PTC and 17% of MTC cases and anti-COPS4 autoantibodies in 3% of PTC cases but not in the patients with benign nodules and cancer-free controls, thus suggesting these autoantibodies may serve as highly specific biomarkers for thyroid cancer and may have a clinical utility if combined with other autoantibody biomarkers. However, we did not find autoantibodies against any other Cancer-Testis antigens included in our antigen microarray, suggesting that spontaneous

humoral immune response against common tumor associated antigens is relatively rare in thyroid cancer in comparison with other solid cancers. This probably is due to the relatively indolent nature of PTC and FTC.

In conclusion, this doctoral thesis provided a deeper insight into the molecular alterations leading to the development of thyroid cancer and revealed novel biomarkers for the diagnosis of thyroid cancer.

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Introduction

Thyroid nodules are detected by ultrasound in up to 30 % of women and ~ 6 % men that are over 50 years old. Most of them are benign - hyperplastic nodes or follicular adenoma (FA), which usually does not require radical treatment, but ~ 5% of the nodules are malignant tumors. However, thyroid cancer is the most common endocrine cancer and the incidence rate in the developed countries has increased from 3.6 cases per 100 000 population in 1973 year to 8.7 cases in 2002. For women thyroid cancer occurs 4 to 5 times more frequently than men (Ries LAG et al., 2007). Approximately 90-95% of cancers are well-differentiated papillary (PTC) or follicular (FTC) carcinoma, which are derived from thyroid follicular cells, ~ 3% are medullary carcinomas formed from C cells and 2-7 % are low- differentiated or anaplastic carcinomas that are most likely develop by progression of PTC or FTC. Most well-differentiated thyroid carcinomas are treated with surgery and radioiodine therapy, but in ~ 15% of cases the tumor is very aggressive and become resistant to the drugs. In contrast, low differentiated or anaplastic carcinomas are highly aggressive tumors for which there is currently no effective therapies and patients typically die within the first year after diagnosis (Cerutti et al., 2004;Eszlinger and Paschke, 2010).

Currently, cytological evaluation of fine needle aspiration biopsy is the standard diagnostic method by which malignant tumors are distinguished from hyperplastic nodules and adenomas. However, the cytological examination is based on a subjective interpretation of the results, as well there is a significant impact on the results of the methodology used to sample the biopsy that differs between various clinics. For example, the sensitivity ranged from 43 to 98 % and specificity - from 72 to 100% in various clinical centres (Gharib et al., 2008). In addition, in 2-21 % of cases, biopsy is not informative because of the lack of adequate amount of cells and therefore must be repeated, but in 15-20% it is impossible to distinguish benign from malignant thyroid nodules due to lack of highly specific, measurable cytological criteria (Baloch et al., 1998). Thus, a large proportion of patients with benign nodules is undergoing unnecessary surgery that could be avoided if appropriate pre-operative diagnostic assays were available Therefore, the identification of new molecular biomarkers of thyroid cancer tissue, which, in combination with cytological examination, give the opportunity to improve the accuracy of diagnostic tests and predict the course of the disease is very urgent. Moreover, the identification of biomarkers that are detectable in body fluids, such as plasma or serum, would allow to develop non-invasive diagnostic assays that do not rely on sampling of the nodule by FNA.

Any molecular changes - somatic mutations in the DNA, methylation changes, increased mRNA, miRNA, or protein expression levels, post - transcriptional modifications, that occur in cancer, but not benign nodule tissue can serve as biomarker for the diagnosis of cancer. The most common genetic changes in thyroid tumors are RET or PAX8 gene translocation and BRAF or RAS mutations that are found in up to 70% of cases. However, these mutations are frequently detected in follicular adenoma and even Hashimoto cases (Eszlinger and Paschke, 2010;Nikiforov, 2002;Nikiforova et al., 2002;Xing et al., 2004). The best of the currently known immunohistochemistry markers for diagnosis are galectin -3, HBME -1 and cytoceratin 19, with sensitivity from 78 to 94 % and specificity - from 70 to 93% for each marker, however these results are variable between different studies (Bartolazzi et al., 2008;Park et al., 2010). Several

studies have compared the gene expression profiles yielding more than 100 genes, whose mRNA expression levels differs in benign and malignant thyroid tissue (Barden et al., 2003;Prasad et al., 2005), as well a number of potential new markers has been identified for lymph node metastasis prediction (Cerutti et al., 2004), yet in almost all cases, further studies were needed to evaluate the relevance of these markers.

However, the identification of new tissue biomarkers would not solve the problems associated with the sampling of biopsies (variable cell counts, do not contain all the cell types, etc.). In addition, biopsy is invasive, unpleasant for the patient and also expensive procedure. Therefore, a serological assays for the detection or differential diagnosis of thyroid nodules would be of very high clinical relevance. As a serological marker can serve DNA from circulating tumor cells (mutations or methylation markers), cytokines, chemokines, miRNA, growth factors and autoantibodies against tumor cell antigens.

1. Literature overview

1.1 Cancer development

Cancer is a very heterogeneous disease caused by genetic and epigenetic alterations in oncogenes, tumor-suppressor genes and non-coding RNA genes (Fearon and Vogelstein, 1990;Kanwal and Gupta, 2010). A single genetic change is rarely sufficient for the development of a malignant tumor, in fact most studies point out multistep process of sequential alterations, like chromosomal rearrangement, mutations, gene amplifications in many oncogenes, tumor - suppressor genes and non-coding RNA genes (Fearon and Vogelstein, 1990;Wood et al., 2007). Genetic alterations are irreversible, epigenetic alterations are reversible and do not require direct alterations of DNA sequence. While genetic information provides sequence for protein synthesis, epigenetic information provides instructions on when and where the genetic information will be used. There are several epigenetic mechanisms that are responsible for modifying gene expression and are related to cancer development: DNA methylation, modification of histone proteins and functional non-coding RNA (Foley et al., 2009). The major form of epigenetic regulation in mammalian cells is DNA methylation that is essential for normal embryonic development and has a variety of important functions in cell physiology. Genomic methylation patterns are often altered in cancer cells with global hypomethylation accompanying region-specific hypermathylation sites, usually within the promoter of tumor suppressor genes, that can silence expression of associated gene and provide cell with growing advantage and leading to cancer development (Kanwal and Gupta, 2010).

More recent studies suggest that besides genetic and epigenetic heterogeneity between cancer patients there is also an intratumor heterogeneity. Now based on a single cell genome sequencing we know that tumors often possess different clones that possibly arise from initial transformed or progenitor cancer cells trough genetic alterations all of which constitute a spectrum of cells with different genetic alterations and states of differentiation. Epigenetic alterations and plasticity are likely to have an important role on phenotypic and functional intratumoral heterogeneity. The heterogeneity contributes to differences in clinical behaviour and response to treatment (Bedard et al., 2013;Shah et al., 2012). Besides the clonal cancer evolution model or stochastic model, there is a Cancer Stem Cell (CSC) model that explains this intratumor heterogenecity. This model suggests that cancers arise from self renewing stem cells that are biologically distinct from their more numerous differentiated progeny. They usually express organ specific markers, are resistant to chemotherapy and are able to generate a new tumor in immunodeficient mice. Moreover, there is growing evidence that pathways regulating normal stem cell self renewal and differentiation are also active in CSCs (Yu et al., 2012b). However, most likely both paradigms of tumour development exist in human cancer and it is important to note that the two models are not mutually exclusive, as CSCs can undergo clonal evolution also (Barabe et al., 2007). In the end, lethality of cancer is the ultimate result of deregulated cell signalling and regulatory mechanisms as well as inappropriate host cell recruitment and activity that leads to the generation of niche for cancer cells within tumor that replace normal cell functions creating hypoxic, acidic and nutrient poor environment that in orders increases genetic and epigenetic instability promoting cancer development (Amend and Pienta, 2015).

1.2 Normal thyroid gland development

Mature human thyroid is composed of epithelial cells, endothelial cells and fibroblasts. For example, in mature dog thyroid there is 70% of epithelial cells, 24% endothelial cells and 6% fibroblasts (Dow et al., 1986). In human thyroid, most of epithelial cells are T3 and T4 hormone producing cells or follicular cells, which develop from endodermal cells and only 1% of epithelial cell mass are parafollicular cells or C cells producing calcitonin, which develop from neiroectodermal cells. In the mouse, where thyroid embryogenesis has been well studied, thyroid development starts between embryonic day E8 and E8.5 (in humans ~ 4^{th} week) with the specification of the thyroid anlage, a thickening of the midline endodermal floor of the primitive foregut. The expression of transcription factors TTF1, TTF2, Pax8, HHEX and transcription cofactor TAZ induces follicular cell precursors become polarized and organized in follicular lumens at this stage. Follicular lumens accumulate colloid that protects follicular cells from peroxidise accruing in idonation of thyroglobulin. The expression of these transcription factors and cofactor are also necessary for the maintenance of differentiated phenotype of thyroid follicular cell (Di et al., 2009;Hoyes and Kershaw, 1985). Further, the median anlage develops into a diverticulum that descends caudally and loses contact with the pharynx by E11.5 in mouse (in humans ~ 5^{th} week). After detachment, the thyroid primordium expands bilaterally and migrates to its final position in front of the trachea until E13.5 (in humans ~ 8^{th} week). Terminal functional differentiation of the thyroid around E14.5 (in humans ~ 10^{th} week) involves expression of the functional differentiation markers - at the beginning TSHR that in turn stimulates expression of TG and TPO. The expression of SLC5A5 becomes detectable latter, and functional maturation is evident from thyroid hormone synthesis around E16.5 (in humans ~ 12^{th} week) (De and Di, 2004;Fagman et al., 2006). While source of follicular cell of thyroid embryonal structure origin is thyroid anlage, parafolicular cell source is ultimobranchial bodies. The C cells of the ultimobranchial bodies migrate from their respective sites of origin and ultimately merge in the definitive thyroid gland (in human 8^{th} week). In the merging process, the ultimobranchial bodies disappear as individual structure, and the cells contained in it disperse in the structure of the adult thyroid gland. While the cells originating from the anlage continue to organize the thyroid follicles, the C cells scatter within the interfollicular space (Fontaine, 1979).

1.3 Thyroid cancer development

There is evidence that thyroid cancer is developed in multi step carcinogenesis via genetic and epigenetic alterations that accelerates malignant phenotype development such as increased proliferation, invasion into surrounding tissues and metastasis to distant organs. Advanced biological methods such as karyotypic analysis, fluorescent *in sity* hybridisation, microarray expression analysis and whole genome associations has helped scientists to find out some of the molecular defects causing the development of thyroid cancer. One of these defects is translocations of chromosomes that produce novel proteins with oncogenic properties as a result of fusion of two genes. One of the most frequent fusions is generated by translocation involving thyroid specific transcription factor PAX8 (chromosome 2q13) and PPARγ nuclear hormone receptor (chromosome 3p25) that is normally involved in the differentiation of various tissues, especially adipocytes. This rearrangement has been identified as an early event in the development of thyroid cancer. There are several hypotheses how this fusion gene contributes to

oncogenesis. There is evidence that this fusion protein can act as a dominant negative suppressor of PPARγ induced gene expression that contributes to anti apoptotic properties (Kroll et al., 2000), while other studies that show that this fusion leads to the disruption of PAX8 transcriptional activities resulting in decreased expression of thyroid specific genes (Au et al., 2006). Another known gene rearrangement involved in thyroid cancer development is RET rearrangement in which tyrosin kinase domain of the normally silent RET is fused with various constitutively expressed genes. There are at least 10 types of rearrangements but the most common products are RET/PTC1 (inv(10)(q11;2q21)) and RET/PTC3 (inv(10)(q11;2q10)). Both involve inversion of the long arm of chromosome 10, generating a fusion between RET and either histone H4 (histone protein in nucleosome) or nuclear receptor coactivator 4 (NCOA4) gene (Zitzelsberger et al., 2010). Another frequent rearrangement involves an inversion of chromosome 7q generating fusion between BRAF and AKAP9 (A-kinase anchor protein 9 gene) containing BRAF kinase domain without the N-terminal auto-inhibitory domain in radiation induced thyroid carcinomas (Ciampi et al., 2005). Another rearrangement involves the fusion between the 3' terminal sequences encoding the kinase domain of NTRK1 (neutropic tyrosine kinase receptor type 1) on chromosome 1 and 5' terminal sequences of various genes resulting in activated TRK oncogenes (Greco et al., 2010). All these fusions leads to constitutive activation of the MAPK pathway that induce expression of genes like, Myc, estrogen receptor, cyclin D1 that leads to inhibition of normal thyroid cell differentiation and increasing cell proliferation and survival (Kouniavsky and Zeiger, 2010).

Another type of genetic alterations in thyroid cancer are somatic mutations and the most frequent one is BRAF nucleotide substitution from A to T, which change valine to glutamic acid at amino acid position 600. The change activates BRAF kinase leading to stimulation of MAPK pathway (Kimura et al., 2003). Interestingly, several studies show that thyroid cancer cells harbouring BRAF mutation often have methylation of iodide metabolising genes. Treatment of follicular cell derived thyroid cancer cell lines harbouring this mutation with MEK (mitogen activated protein kinase 1) inhibitor restored SLC5A5 (NIS) and TSHR expression, suggesting that this mutation is responsible for SLC5A5 and TSHR promoter methylation and thyroid tumorigenesis (Akagi et al., 2008;Liu et al., 2007). In MAPK pathway upstream of BRAF there is a kinase RAS that have three forms: H-RAS, K-RAS and N-RAS. Mutations that always localize to either codon 12, 13 or 61 of RAS forms constitutively activates the MAPK and PI3K/Akt signaling pathways. PI3K/Akt signaling pathway in turn activates expression of genes like NF- kB, Mdm2, YB-1 and inhibits expression of p21, p27, BAD, Casp9, leading to thyroid cancer cell resistance to apoptosis, proliferation, survival and migration (Vasko et al., 2003). PTEN is phosphotase that acts as a suppressor of PI3K/Akt pathway. Both deletion of the PTEN locus (10q23) and silencing of PTEN by aberrant promoter methylation enhance PI3K/Akt signaling and is associated with the progression of thyroid tumor (Alvarez-Nunez et al., 2006;Yeh et al., 1999).

Increasing evidence suggests that mutations or polymorphisms in non-coding RNA also can contribute to development of thyroid cancer. For example polymorphisms in short non-coding RNA miR 146a precursor (pre-miR 146a) cause decreased amount of mature miR-146a that reduce inhibition of its possible target genes IRAKI1 (IL1 receptor associated kinase 1) and

TRAF6 (TNF receptor associated factor 6) – the key molecules in TLR (tool like receptor) pathway, suggesting that patients carrying this polymorphism have predisposition to PTC, because PTC have increased TLR activity and increased cytokines and chemokine level (Jazdzewski et al., 2008).

Another pathway involved in thyroid cancer development is β-catenin signaling. β-catenin is a part of a cytoplasmic complex regulated by GSK3β (glycogen synthase kinase-3). When the complex is phosphorylated by GSK3β, it undergoes ubiquitination and degradation. PI3K/Akt pathway induces phosphorylation of GSK3β that in turn releases β-catenin from the complex allowing it to be translocated to nucleus. In the nucleus, β-catenin activates TCF/LEF (T-cell-specific transcription factor/lymphoid enhancer binding factor) target genes such as cyclin D1 and Myc promoting cell proliferation and survival. In some thyroid cancers β-catenin can contain a mutation in exon 3 where the phosphorylation sites are involved in β-catenin degradation and that is associated with aberrant nuclear localization and poor prognosis (Garcia-Rostan et al., 2001).

Advances in molecular biology methods provided us with information on the genetic and epigenetic alterations such as BRAF, RAS and β-catenin mutations, RET/PTC, PAX8/PPARγ, rearrangements, PTEN locus deletion and aberrant promoter methylation, SLC5A5 and TSHR promoter methylation, that can lead to thyroid cancer development. This information can improve the differential diagnostics and treatment of thyroid cancer. Even more, in the last decade there has been a great effort to identify gene expression signatures of downstream genes that could improve diagnostic value of molecular diagnostic tests and possibly identify new molecular pathways of thyroid cancer development.

1.4 Thyroid cancer diagnosis and molecular biomarker identification

Relevance of molecular biomarkers for differential diagnostics of thyroid cancer

Artūrs Ābols
Latvian Biomedical Research and Study center; University of Latvia
Rīga, Latvia

Kristīne Ducena
University of Latvia
Rīga, Latvia

Aija Linē
Latvian Biomedical Research and Study center
Rīga, Latvia

Valdis Pīrāgs
Pauls Stradiņš Clinical University Hospital; University of Latvia
Rīga, Latvia

1 Diagnostics of thyroid cancer

Thyroid nodular disease is a common clinical finding. The prevalence of palpable nodules ranges from 3 to 7% in the general population, with an incidence of approximately 1 case in 1000 individuals per year (Gharib, Papini, & Paschke, 2008; Ries LAG, Melbert D, & Krapcho M, 2007; Parkin, Bray, Ferlay, & Pisani, 2005). Asymptomatic thyroid nodules are detected up to 65% on ultrasound and ~50% in pathologic examination of autopsy studies in North America. Although only less than 5% of the palpable nodules are malignant lesions, however thyroid cancers are the most common malignancy of endocrine organs.

The etiology of thyroid tumors is multifactorial, including environmental, genetic and endogen hormonal factors. Iodine deficiency is the major environmental factor, contributing the development of both endemic and sporadic nontoxic nodular goiter. The evidence of genetic predisposition is best established for medullary carcinoma and approximately 25% of these tumors are involved in multiple endocrine neoplasia syndromes: MEN 2A, MEN 2B and familial medullary carcinoma (FMTC).
Thyroid tumors may manifest in patients with well-known hereditary genetic syndromes like familial adenomatous polyposis (FAP) and Cowden disease and as well as in cases of rare genetic syndromes, including Carney complex, Werner syndrome, Peutz-Jegers syndrome and MEN1 syndrome (Charkes, 2006; Charkes, 1998).

The role of endogenous hormonal factors is based on evidence of significant prevalence of thyroid tumors in woman. Some studies have found that the increased risk of thyroid cancer is associated with use of estrogen containing oral hormonal contraceptives. This hypothesis is supported by the fact that estrogens interact with the mitogen-activated protein kinase (*MAPK*) signaling pathways which play a significant role in proliferation and differentiation of thyroid cancer (Chen, Vlantis, Zeng, & van Hasselt, 2008; Manole, Schildknecht, Gosnell, Adams, & Derwahl, 2001).

The examination of patient with newly discovered thyroid nodules starts with complete evaluation of the history of disease and the basic physical examination, including inspection of regional lymph nodes. The clinical risk factors indicating for thyroid malignancy include history of head and neck irradiation in the childhood, total body irradiation for bone marrow transplantation, family history of thyroid carcinoma or genetic syndromes, including Cowden's syndrome, Carney complex, familial polyposis, MEN 2 syndrome, Werner's syndrome, as well as exposure to ionizing radiation in childhood or adolescence and a rapid growth of a nodule. Physical symptoms suggesting for the thyroid malignancy include a firm consistence of a nodule, hoarseness, vocal cord paralysis, lateral cervical lymphadenopathy, fixation of the nodule to surrounding tissue (Gharib et al., 2008; Hegedus, 2010).

1.1 Serum thyrotropin measurement

Serum thyrotropin (TSH) measurement is the most informative laboratory test. Patients with normal or elevated TSH level should undergo ultrasound imaging, but those with the suppressed TSH level additionally should perform radionucleotide scan, as it likely may be a signal of toxic adenoma (Boelaert et al., 2006; Gharib et al., 2008; Hegedus, 2010).

Several studies also show that TSH can serve as a thyroid tumor marker. For example, Bolaert and colleagues demonstrated that the risk of malignancy increases if the patient's TSH is >0,9 mIU/I. Binary regression analyses demonstrate that patients whose TSH is between 1,0 – 1,7 mIU/I have a greater risk

to develop thyroid cancer than patients whose TSH is lower than 0,4 mIU/I, but the risk is even greater if the patient's TSH is between 1,8 – 5,5 mIU/I (Boelaert et al., 2006).

However, some reports accent limitations of the use of TSH for malignancy risk evaluation. For example, patients with a nodular goiter referred for thyroid surgery do not represent the average population. It should be noted that the level of TSH differs among various racial and ethnic groups as well as during the aging process. For example, Caucasians show a higher overall concentration of TSH, while older people (>80 years) show a higher level of TSH compared with individuals aged 20 – 29. These data lead one to surmise that TSH *per se,* increases the possibility of tumor development in thyroid nodules as patients get older (Jin, Machekano, & McHenry, 2010).

1.2 Evaluation of thyroid nodules with ultrasonography

High resolution ultrasonography (US) is the most commonly used imaging method for evaluation of thyroid nodules. US allow detecting small non palpable nodules, although the malignancy risk is the same comparing with the palpable nodules of the same size. Nearly 17-67% of randomly selected population has thyroid nodules at US and majority of them does not cause serious health problems (Hegedus, Bonnema, & Bennedbaek, 2003; AACE/AME, 2006; Hegedus, 2010).

Guideline accepted US features associated with thyroid malignancy include: microcalcifications, marked hypoechogenity, irregular or blurred margins, nodule taller than wide, absence of halo and increased intra-nodular vascularity (Frates et al., 2005; Hoang, Lee, Lee, Johnson, & Farrell, 2007; Kim et al., 2008; Peccin et al., 2002).

Microcalcifications are psammoma bodies, ~10-100μm round laminar crystalline calcific deposits and in US they appear like punctuate hyperechoic foci without acoustic shadowing (Hoang et al., 2007). It is the most specific US feature of malignancy, with a specificity of 85.8-95% and a positive predictive value of 41.8-94,2 in the large population studies. Microcalcifications are found in 29-59% of all primary thyroid carcinomas, mostly in the cases of papillary thyroid carcinoma (Frates et al., 2005; Hong et al., 2010; Kim et al., 2008; AACE/AME, 2006).

Capelli C et al. analyzed US of 7445 nodules (diameter 6-100mm) from the period of 1991 till 2004 and verified that presence of microcalcifications is highly associated with the presence of thyroid cancer in surgery material comparing with the benign findings (72,2 vs. 28,7%, $p<0,001$). Coarse or macrocalcifications are more specific to benign thyroid nodules, especially with long duration of disease (Cappelli et al., 2007), although can appear together with microcalcifications in papillary thyroid carcinomas, and more often in the cases of medullary thyroid tumors (Hoang et al., 2007; Takashima et al., 1995).

Marked hypoechogenicity of the nodule comparing with the surrounding normal thyroid parenchyma is very suggestive for malignancy. Malignant thyroid nodules typically appear like solid hypoechoic formations and combination of microcalcifications and marked hypoechogenicity have sensitivity as high as 87%, but low specificity (15.6%-27%) and positive predictive value for malignancy. In a large retrospective study Moon et al. analyzing more than 8000 patients, hypoechogenicity was found in 33, 7% of all benign nodules and 46,1% of malignant nodules (Moon et al., 2008). Most of the studies emphasize that exactly marked hypoechogenicity is highly suggestive for malignancy than hypoechogenicity assessed comparing with surrounding thyroid tissue (Frates et al., 2005; Hoang et al., 2007; Kim et al., 2008; AACE/AME, 2006).

Thyroid nodule is defined as irregular in the cases if >50 % of the border is not clearly demarcated. The average reported sensitivity for this US feature is very wide (7%-97%) and the specificity ranges from 15-59%. A large population study of Capelli et al demonstrated **irregular or blurred margins** in 52,8% cases of malignant nodules comparing with 18,8% of cases in benign ones (P<0,001) (Cappelli et al., 2007).

Shape taller than a wide is also well-known US predictor of malignancy. Capelli et al found if a nodule with shape taller than wide (anteroposterior/transverse diameter ratio ≥1, A/T ≥1) together with two other specific US features (microcalcifications, blurred margins, hypoechoic pattern) is the best model for predicting malignancy. In this study A/T ≥1 was significantly more frequent in malignant lesion than in benign nodules (76% vs 40%, p<0,001), but the combination with another two US features demonstrates high diagnostic value (sensitivity of 99%, specificity 57%, PPV 6,0%, NPV 99%, missed carcinoma 0,9%) (Cappelli et al., 2006; Cappelli et al., 2005).

A halo zone is a hypoechoic rim around a thyroid nodule and is very characteristic to benign nodules with the specificity up to 95%, although 20-24% of all PTC have a partial or distinct halo zone (Frates et al., 2005; Hoang et al., 2007; Kim et al., 2008; AACE/AME, 2006). Some studies emphasize, that halo is absent more than half of benign nodules (Hoang et al., 2007; Propper, Skolnick, Weinstein, & Dekker, 1980).

Increased intranodular vascularity of the nodule, detected by using color Doppler, is well known predictor of thyroid malignancy comparing with benign nodules that present peripheral vascular pattern.(AACE/AME, 2006; Alexander, 2008; Frates et al., 2005; Peccin et al., 2002). Study of Frates et al. confirmed that solid hypervascular nodules possess higher malignancy risk (Frates, Benson, Doubilet, Cibas, & Marqusee, 2003). Also Papini et al. reported intranodal vascular pattern in 74,2% of malignant nodules and it serves as an individual risk factor (Papini et al., 2002). Capelli et al found intranodal vascularity in 61.6% of malignant nodules and also associated it with increased cancer risk. In the opposite, completely vascular nodules rarely are malignant(Cappelli et al., 2007).

Local invasion and lymph node metastases are highly specific to malignancy. Extracapsular invasion has been demonstrated in 36% of thyroid malignancies in postoperative histological analyses (Alexander, 2008; Hoang et al., 2007; Papini et al., 2002). It is known that differentiated thyroid carcinoma (mainly papillary) involves cervical lymph nodes in 20-50% of patients and may be present even when the primary tumor is small and intrathyroidal. Metastases in regional lymph nodes correlate with the further prognosis and recurrence of the disease (Frates et al., 2005; Hoang et al., 2007; Kim et al., 2008; Peccin et al., 2002; AACE/AME, 2006).

Some studies are aimed to evaluate US malignancy features especially for non-palpable nodules. Study of Papini et al. has evaluated the risk of malignancy of small non-palpable thyroid nodules with diameter 8-15mm and they found that cancer prevalence was similar both in nodules greater or smaller than 10mm (9.1 vs. 7.0%) This study demonstrated that significant majority of cancers appeared at US as hypoechoic structures (87% of cases) and the independent US risk features of malignancy in US were irregular or blurred margins (77,4%), increased intranodular vascularity (74,2%) and microcalcifications (29%) (Papini et al., 2002). The study of Kim et al evaluated 1325 non-palpable thyroid nodules in 1009 patients from which FNA cytology revealed 823 benign, 154 indeterminate, 198 nondiagnostic and 150 malignant nodules. 58 malignant nodules and 82 benign nodules were included for the further analysis, were 3 radiologists analyzes the echogenity, shape, margins, calcifications, degree of cystic changes and the size of nodules. Results showed that marked hypoechogenicity, an irregular shape, a taller than shape,

a well-defined speculated margin, microcalcification and entirely solid nature were significant predictors for malignancy ($p<0,05$), whereas a cutoff value of 1 cm in longest diameter was not significant ($p<0,184$) (Kim et al., 2008).

US findings like size of nodule, interval growth of nodule and number of nodules are not helpful for predicting or excluding thyroid malignancy (Hoang et al., 2007; Jun, Chow, & Jeffrey, 2005).

Considering that US is commonly used diagnostic tool for assessing thyroid malignancy, sensitivity, specificity, positive and negative predictive values ranges widely in reported studies. Park et al described average inter observer variability and concluded that the sensitivity, specificity, PPV and NPP were 65,3%-81,9%, 60,7%-68,9%, 69,7%-73,8% and 66,6-75,5%, respectively (Park et al., 2010). Choi SH evaluated four radiologists with more than 5 years' experience in thyroid US, analyzing 204 thyroid nodules (89 benign and 115 malignant). Echogenity, calcifications, composition, margins, shape and vascularity were analyzed with the final assessment. Inter and intra-observer variability were detected with Cohen's kappa statistics and accuracy was calculated. Overall sensitivity, specificity, PPV, NPV and diagnostic accuracy were 88,2%, 78,7%, 76,2%, 98,6% and 82,8% for malignancy risk and conclusions were done with high accuracy in final assessment (Choi, Kim, Kwak, Kim, & Son, 2010). The study of Hong YJ evaluated the PPV of suspicious sonographic features and the results demonstrated that PPV for microcalcifications were 38,6%, for irregular margins 28,2%, for marked hypoechogenicity 49.4%, for nodule taller than wide 59,8% and concluded that three US features: microcalcifications, marked hypoechogenicity and a shape taller than wide are the best predictors of malignancy (Hong et al., 2010). One of the reasons for wide inter-observer variability could be explained by fact that thyroid US is done by different specialists with different US experience. Interesting study of Kim at al reported inter-observer variability in the interpretation of US findings of thyroid nodules and authors concluded that final agreements between five academic faculty radiologists were fair-to-good, but poor-to-fair between four residents of radiology (Kim, Kwak, Kim, Choi, & Moon, 2012). Finally, although inter-observer variability can differ between studies, the majority of reported findings suggests that microcalcifications, irregular or blurred margins, marked hypoechogenicity, increased nodal vascularity and abnormal neck lymphadenopathy are associated with higher malignancy risk (Alexander, 2008; Choi et al., 2010; Frates et al., 2005; Hoang et al., 2007; Hong et al., 2010; Kim et al., 2012; Kim et al., 2008; Peccin et al., 2002; AACE/AME, 2006).

1.3 Ultrasound elastography

As mentioned above, palpation is a basic clinical examination method of thyroid nodules. Benign nodules are usually firm on physical examination; however malignant nodules turn to be much harder. Ultrasound elastography provides direct information corresponding to a hardness of nodule. It is a dynamic technique which estimates the tissue stiffness by measuring the degree of distortion under the application of external force and is based on the evidence that softer nodules have a greater elastic strain comparing with harder nodules of no strain. Using a numeric score system (graded 1-5), US elastography has been successfully used for differentiation benign nodules from malignant (Alexander, 2008; Rago, Santini, Scutari, Pinchera, & Vitti, 2007; Rago et al., 2010). Study of Rago T et al. evaluated 92 patients who underwent thyroid surgery due to compressive symptoms of suspicion of malignancy on FNA cytology. The results showed scores 1 and 2 were found in 49 cases (all benign nodules), score 3 in 13 cases (12 benign, one malignant) and scores 4 and 5 in 30 cases (all carcinomas) with a conclusion that elasticity scores 4-5 were highly predictive for malignancy ($p<0.0001$) with a sensitivity of 97%, specificity of 100%, PPV of

100% and NPV of 98%. Another study of Rago T et al. included 176 patients with indeterminate or non-diagnostic cytology on FNA and a total of 195 nodules were evaluated and elasticity was scored as 1 (high), 2 (intermediate) and 3 (low). Results demonstrated that in indeterminate lesions, score 1 was highly associated with benign nodule (103 cases, 102 benign, 1 carcinoma, $p<0.0001$), score 2 was found in 14 cases (8 benign nodules and 6 carcinomas) and score 3 was found in 25 cases (1 benign, 24 carcinomas). In cases of non-diagnostic cytology, score 1 was found in 40 cases (39 benign lesions, 1 cancer), score 2 in four nodules (3 benign, 1 carcinoma) and score 3 in 9 nodules (six carcinomas, three benign nodules) with conclusion that US elastography is a useful tool in cases of indeterminate and non-diagnostic cytology for selecting candidates with potential need of thyroid surgery (Rago et al., 2010). The study of Hong Y el al. included 90 patients with thyroid nodules who were referred for surgery and 145 nodules were evaluated by B-mode US, colour Doppler ultrasound and US elastography and tissue stiffness was scored from 1 (low stiffness) to 6 (high stiffness). Results showed that on real-time US elastography, 86 of 96 benign nodules (90%) had a score 1-3, while 43 of 49 malignant nodules (88%) had a score 4-6 ($p<0.001$), with sensitivity of 88%, specificity of 90%, a PPV of 81% and a NPV of 93% with conclusion that US elastography is a useful imaging technique for differential diagnosis of thyroid cancer (Hong et al., 2009). As US beam is unable to cross calcified structures, the main limitations for US elastography are nodules having calcified shells or rims, partially or completely pure cystic nodules and in cases of multinodular goiter. Recently a new shear wave elastography (SWE) that estimates tissue stiffness in real time and user independent has been developed. The quantitative information is expressed as an elasticity index (EI) and expressed in kilopascal (kPa) on continuous scale without applying external force (Hegedus, 2010; Sebag et al., 2010). The study of Sebag et al assessed the efficiency of SWE and compared it with high resolution US, evaluating 146 nodules from 93 patients, from those 29 nodules (19.9%) were malignant. Mean (±SD) EI was 150±95kPa in malignant nodules vs. 36±30 kPa in benign nodules ($p<0.001$), PPV 80%, sensitivity 85,2%, specificity 83.97%. Comparing with US score, reported sensitivity was 51.9%, specificity 97%, but in combination of US +SWE, sensitivity raised to 81%, specificity 97% (Sebag et al., 2010).

In summary, although conventional US and US elastography are highly predictive, still all thyroid guidelines recommend to do FNA as universal agreement for assessing malignancy risk of thyroid nodules (Hegedus, 2010).

1.4 Cytological evaluation of FNA

Fine-needle aspiration (FNA) currently is the most widely used preoperative method for evaluation of thyroid nodules. FNA is cost effective, minimally invasive and safe procedure, although it has limitations for certain types of tumors.

In October 2007, The National Cancer Institute (NCI) reviewed the algorithms for the use of FNA in the management of thyroid nodules and summarized the indications of FNA, pre-FNA requirements, diagnostic terminology, post FNA testing and treatment options (Baloch et al., 2008; Kim et al., 2008; Layfield, Cibas, Gharib, & Mandel, 2009).

FNA with following cytopathology is recommended for most of patients with normal or elevated TSH level and thyroid nodules>1-1,5 cm, detected by palpation or thyroid US, unless they are simple or separated cysts with no solid elements. The functioning thyroid nodules do not require FNA in the absence of significant clinical or US findings. The same indications for FNA are also related for incidentally found thyroid nodules. The American Association of Clinical Endocrinologists recommended FNA

even for nodules smaller than 10mm for patients with suspicious clinical or US features of malignancy. Traditionally, one of the main indications for FNA was the presence of a solitary nodule (Baloch et al., 2008; Kim et al., 2008; Layfield et al., 2009).

In the recent years, the large clinical observations indicate that patients with the multiple nodules have the same malignancy risk as a solitary nodule. It means that the presence of suspicious US features is the main indication for performing FNA, so all the nodules >1cm, with microcalcifications, marked hypoechogenity, irregular or microlobulated margins, intranodal vascularity, a shape taller than wide and extracapsular invasion should undergo FNA (Baloch et al., 2008; Kim et al., 2008; Layfield et al., 2009).

Pre-FNA requirements include informed consent form and the discussion of procedure with patient, including the information about potential risks and complications (slight local pain and minor hematoma). The techniques for FNA are widely described in the literature. Traditionally, for US guided FNA the patient is placed in a supine position with the neck slightly extended. A local anesthesia with 1-2 ml of 1% lidocaine hydrochloride solution is injected at the predeterminate site of biopsy. Commonly available25-27 gauge needles are used together with injection syringe, with the exceptions of cysts with viscous contents when the larger needle sizes (22-23 gauges) are needed (Hamburger, Husain, Nishiyama, Nunez, & Solomon, 1989; Kelly et al., 2006; Wang, 2006; Baloch et al., 2008).

It is strongly recommended that aspiration should be performed at least twice from the suspicious nodule, each from the different area of the nodule. The FNA specimen is directly smeared on glass slides for air dried or alcohol fixed preparations, stained by Romanowsky or Papanicolaou techniques, respectively. Papanicolaou staining is the most commonly used for the cytological analysis, demonstrating the clearest description of nuclear changes of the thyroid cells (Baloch et al., 2008; Hamburger et al., 1989; Kelly et al., 2006; Layfield et al., 2009; Wang, 2006).

FNA sample is considered adequate for the interpretation, if the sample contains a minimum of 6 clusters of at least 10 follicular epithelial cells on two or more slides. Aspirates are considered as non-diagnostic, if the material is poorly fixed, prepared and stained or contains only cyst fluid or peripheral blood cells.

Different cytological classification systems are used in the clinical practice depending from the professional societies. The most commonly used is Bethesda System, containing of six following categories (Baloch et al., 2008; Kim et al., 2008; Layfield et al., 2009):

- Benign – low risk of malignancy, <1%. The category includes adenomatoid nodules (with the variable amount of colloid or increased number of follicular epithelial cells), chronic lymphocytic thyroiditis, non-neoplastic nodular goiter.
- Atypia of undetermined significance (AUS) or follicular lesion of undetermined significance (FLUS), the risk of malignancy 5-15%. Very marked variability in use; the cytological findings are not convincingly benign, but the cellular atypia is insufficient for the diagnosis of malignancy. The main clinical dilemma: repeat FNA/clinical follow up or lobectomy.
- Follicular neoplasm or suspicious for follicular neoplasm (FN/SFN) and Hürtle cell neoplasm or suspicious for Hürtle cell neoplasm, low to intermediate risk of malignancy 20-30%. The majority of studies demonstrate that up to 20% of follicular neoplasms are found to be malignant in postoperative histology and risk of malignancy is >20% for Hürtle cell neoplasms.
- Suspicious for malignancy (SMN). The category mostly includes cases suspicious for papillary carcinoma and in the majority of cases (50-75%) follicular variant of PTC is founded. The category also applies to the cases suspicious for medullary carcinoma (cases when FNA sample is in-

sufficient to perform confirmatory immunohistochemical staining for calcitonin), suspicious for other primary or secondary malignancies and suspicious for anaplastic carcinoma (total necrosis of lesion cells).

- Malignant. The FNA specimen confirms the diagnosis of papillary, medullary, anaplastic or metastatic carcinoma. This category is associated with the false –positive rate of less than 1%.
- Nondiagnostic or unsatisfactory. The FNA specimen of too few cells for diagnosis or is poorly fixed or contains excessive peripheral blood cells.

Despite high diagnostic accuracy of FNA, it has limitations, especially for diagnosis of follicular lesions. Currently a follicular adenoma cannot be distinguished from follicular carcinoma in FNA cytology and patients with follicular neoplasm still undergo diagnostic surgery for this reason (Alexander, 2008; Deveci, Deveci, LiVolsi, & Baloch, 2006; McHenry & Phitayakorn, 2011; Prasad et al., 2005; Scognamiglio, Hyjek, Kao, & Chen, 2006; Bussolati, Gugliotta, Volante, Pace, & Papotti, 1997). The ratio of follicular adenoma to carcinoma in surgical specimens is approximately 5 to 1. The differential diagnosis of follicular adenoma usually includes dominant hyperplastic nodule, follicular carcinoma and encapsulated follicular variant of papillary carcinoma. The main histological difference is capsular or vascular invasion, however, a slight vascular invasion can be found in benign follicular tumors as well. In addition, both tumor types are characterized by various degrees of cellular atypia and vascular invasion and some studies report that minimally invasive follicular carcinoma is a less aggressive tumor with a similar risk of prognosis as benign follicular adenoma. Invasive follicular carcinoma has vascular invasion beyond the tumor capsule and it is associated with a worse prognosis. FNA cytology from patients both with follicular adenoma and follicular carcinoma reveals abundant follicular epithelial cells, microfollicle formation and typically very little or no colloid. A group of patients with follicular tumors have FNA cytology with follicular cells with abnormal architecture and atypia and according to the Bethesda classification system, they are categorized as „atypia of undetermined significance" or „follicular lesion of undetermined significance", with the 5-15% malignancy risk (Prasad et al., 2005; Scognamiglio et al., 2006; Serra & Asa, 2008; van Heerden et al., 1992; Sanders & Silverman, 1998).

In addition, up to 30% of FNA is classified as indeterminate, but only 10-15 % of them prove to be malignant in post-operative histological evaluation and the large majority of patients with indeterminate or atypical cytology undergo thyroid surgery as a diagnostic procedure. The indeterminate categories include: Atypia of undeterminate significance/follicular lesion of undetermined significance (AUS/FLUS), follicular (Hurtle) neoplasms and cases suspicious for carcinoma. Some uncertainties still exist, concerning necessity to repeat FNA in indeterminate cases. Some centers suggest that a larger sample provides better analysis of cellular processes, while others debate that a benign finding in repeated aspiration may provide false assurance of benignity. AACE/AME guidelines consider surgical excision as the best management and argue that repeat biopsy is not helpful and even lead to confusion, while ATA guidelines suggest using molecular markers and a radioiodine scan, to exclude thyroid hyperfunction, in cases of suspicious cytology. Many controversies exist regarding the amount of surgery in indeterminate cases. AACE/AME guidelines recommend thyroid surgery, without specifying the extent or surgery, while ATA suggest lobectomy for a solitary indeterminate nodule, whereas ETA recommends lobectomy for solitary nodule and a near-total thyroidectomy for multinodular goiter (Alexander, 2008; Baloch, Sack, Yu, Livolsi, & Gupta, 1998).

In conclusion - cytological evaluation of FNA is currently the major diagnostic tool, however large proportion of patients with nodules with indeterminate cytology would benefit from the application of molecular biomarkers that could improve the diagnostic accuracy and would avoid unnecessary surgery.

2 Molecular biomarkers of thyroid cancer

An ideal molecular biomarker or a panel of biomarkers should differentiate thyroid nodules into subtypes or at least discriminate benign from malignant nodule in any lesion that is considered as suspicious or indeterminate to improve thyroid cancer diagnosis and treatment. The assay should be reproducible, cost-effective and simple to apply. In past decades there has been a great effort to identify such molecular biomarkers that would meet these requirements. The development of the high throughput technologies have provided us with many new potential candidates that are very promising. Here we try to give an insight into the various types of molecular biomarkers, discuss their diagnostic value and the possibilities to exploit them as auxiliary biomarkers for improving FNA accuracy or serum biomarkers for developing non-invasive diagnostic tools (see Table 1).

2.1 Somatic mutation biomarkers

So far, the greatest attention has been paid to detecting the cancer-associated somatic mutations in FNA biopsies. One of the most studied somatic point mutation in thyroid cancer is *BRAF* (V-raf murine sarcoma viral oncogene homolog B1) V600E mutation that leads to the constitutive activation of *BRAF* kinase and stimulation of MAPK pathway that is tumorigenic for thyroid cells (Knauf et al., 2005). This mutation is most common genetic alteration in PTC. *BRAF* V600E mutation is present in 40–50% of classical PTC tissues, 20–25% of FVPTC tissues but only 1,4% of FTC tissues (Chiosea et al., 2009; Kebebew et al., 2007). A number of investigators have evaluated a *BRAF* mutation analysis in FNA. For example, Jo and colleagues prospectively evaluated *BRAF* V600E mutational analysis in 101 patient thyroid nodule FNAB samples (43 benign, 30 malignant, 24 indeterminate or suspicious, 4 nondiagnostic) by applying pyrosequencing. Thyroidectomy was performed in 54 patients with malignant/indeterminate nodules. 22 malignant, 7 indeterminate nodules and one patient with a nondiagnostic nodule was *BRAF* positive. All *BRAF* positive nodules, including the one nondiagnostic and seven indeterminate nodules, were PTC on final histopathology, yielding a sensitivity, specificity and NPV for PTC of 75%, 100% and 85,9% respectively. However in different studies these data varies in sensitivity from 42 to 84%, in specificity 97-100% and in NPV 17,9 - 86% (Bentz, Miller, Holden, Rowe, & Bentz, 2009; Jo et al., 2009; Kim et al., 2008; Marchetti et al., 2009; Nam et al., 2010). These different results could be explained with different size of sample groups, sample heterogeneity and different methods that were applied to detect *BRAF* V600E mutation.

There is also data that mutations in *RAS* (Rat sarcoma) gene family (*H-RAS, N-RAS* and *K-RAS*) that stimulate both the MAPK and phosphatidylinositol-3-kinase (*PI3K*)/*Akt* pathways is found in 20-40% of thyroid cancer and mostly in FTC and FVPTC (follicular variant of papillary thyroid cancer) (Xing, 2010). However these mutations are also present in 20-40% of FA (follicular adenoma) and other benign nodules, but there is some evidence that *RAS* - mutated FA may be precursors of FTC (Alexander, 2008; Esapa, Johnson, Kendall-Taylor, Lennard, & Harris, 1999; Namba, Rubin, & Fagin, 1990).

The most common chromosomal rearrangements in thyroid cancer are the intrachromosomal rearrangements, *RET/PTC1* and *RET/PTC3*. Fusion of proto-oncogene *RET* with *RFG/ELE1* gene leads to the production of hybrid gene *RET/PTC* (Rearranged in Transformation/Papillary Thyroid Carcinomas) that encodes for tyrosine kinase with increased activity that also constitutively activates *MAPK* pathway. This translocation is present in approximately 50% of PTC and also in benign nodules, but its prevalence is highly variable due to genetic heterogeneity of population that differs between geographical location (Nikiforov, 2002; Tallini & Asa, 2001), so this marker alone cannot be used as marker for malignancy. In contrast to *RET/PTC* rearrangement PAX8/PPAR gamma fusion results from an interchromosomal translocation where paired box 8 (*PAX8*) gene from chromosome 2q13 encoding a transcription factor that has a role in thyroid development, joins with the gene encoding peroxisome proliferator activated receptor γ (*PPARγ*) from chromosome 3p25, (Eberhardt, Grebe, McIver, & Reddi, 2010). *PAX8/PPARγ* is found in 30–40% of conventional-type follicular carcinomas and in a small fraction of follicular adenomas and occasionally in the follicular variant of papillary carcinoma (Nikiforova, Biddinger, Caudill, Kroll, & Nikiforov, 2002).

Initial studies centered mostly on the detection of a single mutation, while more recent studies have analyzed various combinations or panels of mutations (Cantara et al., 2010; Ohori et al., 2010). Non-overlapping constitutive activating mutations of *RET, RAS*, and *BRAF* - which are principle initiators of thyroid cancer by stimulating *MAPK* and (*PI3K*)/*Akt* pathways, and *PAX8/PPARγ* fusion are present in 70% of thyroid cancer (Bhaijee & Nikiforov, 2011)

Yuri E. Nikiforov and group in the end of 2011 published a research where they prospective analyzed *BRAF* V600E, *NRAS* codon 61, *HRAS* codon 61, and *KRAS* codons 12/13 point mutations and *RET/PTC1*, *RET/PTC3*, and *PAX8/PPARγ* rearrangements in 967 consecutive thyroid FNA samples (AUS/FLUS - 653 samples, FN/SFN - 247 samples, and SMC - 67 samples) from 729 patients, that to our knowledge is the biggest patient cohort with indeterminate cytology where somatic mutations were analyzed till now. The detection of any mutation in FNA sample conferred the risk of histologic malignancy. 479 patients underwent thyroidectomy, which provided a histopathological diagnosis for 513 FNA samples. Among the histologically confirmed 513 FNA samples (32 malignant and 184 benign nodules in the AUS/FLUS group; 58 malignant and 152 benign nodules in the FN/SFN group; 28 malignant and 23 benign nodules in the SMC group), 61 *RAS*, 17 *BRAF* V600E, one *RET/PTC*, and four *PAX8/PPARγ* mutations were identified. The correlation of mutational analysis in FNA samples and histopathological outcome in specific groups of indeterminate cytology was the following: AUS/FLUS Sn - 63%, Sp - 99%, PPV - 88%, NPV - 94%; FN/SFN Sn - 57%, Sp - 97%; PPV - 87%; NPV - 86%; SMC Sn - 68%, Sp - 96%, PPV - 95%; NPV - 72% (Nikiforov et al., 2011). This study shows that diagnostic panel of mutation is more accurate than single mutation diagnostic tests and can be successfully used for treatment decisions in cases with indeterminate FNA cytology.

2.2 Gene expression biomarkers

Gene expression profiling using cDNA microarrays or serial analysis of gene expression (SAGE) has revealed several hundreds of genes that are differentially expressed between malignant and benign thyroid nodules (Barden et al., 2003; Finley, Arora, Zhu, Gallagher, & Fahey, III, 2004; Finley, Lubitz, Wei, Zhu, & Fahey, III, 2005). A number of them, including *LGALS3, FN1*(fibronectin1) and others that have been shown to be functionally involved in the carcinogenic process, have been validated by qRT-PCR and confirmed at protein level by immunohistochemistry (Alexander, 2008; Finley, Zhu, Barden, &

Fahey, III, 2004; Jarzab et al., 2005). However, several studies have demonstrated that none of these genes individually has sufficient sensitivity and specificity to be exploited as an independent diagnostic biomarker (Cerutti et al., 2004; Cerutti et al., 2007) and there is a great diversity of the results comparing individual genes between studies. Possible source of variability, especially in FNA samples, could be contributed to different number of thyroid follicular cells and adjacent cell population (for example red blood cells, lymphocytes and macrophages) in the sample, variability of RNA quality and the method for normalization of qRT-PCR data. One of the possible ways to acquire higher accuracy would be multiplexing the assay by including a panel of relevant genes. There are many reports which propose gene expression combinations for differential diagnostics of thyroid cancer, but only few of them has been confirmed by other scientists. However majority of these panels contain at least one of the three genes - *FN1*, *LGALS3* and *TFF3* that are often reported as differentiated expressed genes in thyroid cancer (da Silveira Mitteldorf, de Sousa-Canavez, Leite, Massumoto, & Camara-Lopes, 2011; Ducena et al., 2011; Huang et al., 2001; Takano, Miyauchi, Yoshida, Kuma, & Amino, 2005). For example, *FN1, GALE, MET* and *QPCT* are significantly overexpressed in PTC and can discriminate PTC in FNAB cases with indeterminate/suspicious cytology (da Silveira Mitteldorf et al., 2011). *LGALS3* is statistically significant overexpressed in thyroid cancer, but *TFF3* expression is downregulated, and *TFF3* and *LGALS3* expression ratio could discriminate FTC from FA with 72,4% sensitivity and 83,3% specificity (or 80% and 91.5%, when the pathologically questionable cases were excluded) (Takano et al., 2005) or malignant nodules from benign with sensitivity 72,7% and specificity 85,3% (Ducena et al., 2011), but these combinations needs to be tested in FNAB.

Recently, a group from Germany in prospective study analyzed the expression of 6 genes (*ADM3/HGD1/LGALS3/PLAB/TFF3/TG*) in 156 FNAB samples from patients undergoing thyroidectomy by applying qRT-PCR (Karger et al., 2012). The results of molecular diagnostics were compared with the definite histological outcome comprising 7 FTC, 11 PTC, 40 FA, 53 adenomatous nodules and 45 surrounding normal thyroid tissues. Significant expression differences were found for *TFF3*, *HGD1, ADM3* and *LGALS3* in FNAB of thyroid cancer compared with benign thyroid nodules and normal thyroid. Two FNAB marker gene combinations *ADM3/TFF3* and *ADM3/ACTB* allowed the distinction of FA and malignant follicular neoplasia with NPV up to 94% and PPV up to 86%. There were previously reported that these six gene combination per sample allowed correct prediction of benign nodule in 150 thyroid nodule operation specimen (20 FTC, 20 PTC, 6 AC, 52 benign thyroid nodules, and 52 normal thyroid tissues) with sensitivity of 91%, a specificity of 100%, PPV of 100% and NPV of 94% (Karger et al., 2012; Krause et al., 2008). The authors of the study proposed these promising biomarker combinations for multicenter FNAB studies to improve FNAB especially in FTC and FVPTC cases, which is almost impossible to differentiate from FA in cytological evaluation of FNAB.

Alexander EK et al recently reported a large, prospective multicenter validation study of a gene expression classifier developed by *Veracyte*. In this study, the classifier, which is based on the measurement of the expression levels of 167 genes, was applied to classify 265 indeterminate FNA biopsies. These specimens were selected from 4812 fine-needle aspirates collected at 49 clinical sites and had corresponding histopathological specimens that were subjected to central, blinded review. The gene expression classifier correctly identified 78 of the 85 malignant samples and 93 of the 180 benign samples, yielding a: Sn - 92%; Sp - 52%; PPV - 47%; NPV - 93%. Due to the very high negative predictive value, the classifier is best suited to identify benign, rather than malignant nodules. 7 malignant samples (6 PTC, 1 Hürthle cell carcinoma) were misclassified as benign. The authors showed that all 6 PTC samples had

error in sample collection or cellular heterogeneity of the nodule because there was low follicular cell content. In conclusion the authors summarize that this gene expression panel can be used to identify subpopulation of patients with low risk of cancer in a population of patients that otherwise had an operation because of the indeterminate cytology in FNAB (Alexander et al., 2012; Chudova et al., 2010).

2.3 miRNA as biomarkers

MicroRNA (miRNA or miR) are small ~22 nucleotides long non-coding RNA that regulates gene expression usually on posttranscriptional level by binding mRNA 3' UTR which leads to transcriptional repression or mRNA degradation. One miRNA can regulates even several hundreds of protein coding genes and one protein coding gene can be targeted by several microRNAs. They are involved in all cell processes and can act as oncogenes or tumor-suppressor genes (Hussain, 2012). Deregulated microRNA have been reported almost in every type of thyroid cancer, moreover miRNA expression signature differs between thyroid nodule subtypes (Alexander, 2008; Nikiforova, Chiosea, & Nikiforov, 2009; Vriens et al., 2012). Weber with group compared FTC and FA by miRNA microarray analysis and found that *microRNA-197* and *microRNA- 346* were differentially expressed between these two groups with a accuracy of 87% (Weber, Teresi, Broelsch, Frilling, & Eng, 2006). While *microRNA-146b; 221; 222* are significantly overexpressed in PTC compared to other thyroid cancer and nodule subtypes ($p<0,02$) (Chen, Kitabayashi, Zhou, Fahey, III, & Scognamiglio, 2008). *MicroRNA-146b; 221* overexpression and *microRNA-34b; 130b* down regulation correlates with PTC aggressiveness and can be used as prognostic biomarkers (Yip et al., 2011). *MicroRNA-17-92* cluster that contains seven miRNAs (*microRNA-17-3p; 17-5p; 18a; 19a; 19b; 20a, and 92-1*) are overexpressed in AC cell lines and AC lesions compared to adjacent normal thyroid tissues and plays an important role in AC development and could be also a novel target for AC treatment (Takakura et al., 2008). MicroRNA differs even between hereditary MTC and sporadic MTC, for example, *microRNA-183* and *375* are overexpressed (P=0.001; 0.031) and *microRNA-9** is under-expressed (P=0.011) in sporadic MTC versus hereditary MTC. Also overexpression of *microRNA-183 and 375* in MTC predicts lateral lymph node metastases (P=0.001; P=0.001) (Abraham et al., 2011). While there is convincing evidence that microRNA expression signature clearly differs between thyroid nodule subtypes only a few studies till now have tested these microRNAs in FNAB. One of the first study, where seven microRNA (*microRNA-187; 221; 222; 146b; 155; 224; 197*) where tested in 62 FNAB samples was published in 2008. Based on cytological analysis 13 patients had operation (4malignant, 8 atypical cytology) and histopathological analysis revealed 8 malignant (7 PTC and 1 FTC) nodules and 5 hyperplastic nodules. These 7 microRNA were analyzed in all FNA samples without knowing the surgical pathology diagnosis for all samples. Among them all 8 malignant and one hyperplastic FNAB samples revealed more than 2-fold change overexpression of one to six microRNA. 46 of 49 samples from patients that didn't have operation revealed no up-regulation of any of these microRNA and overall performance of the data set resulted in 100% specificity, 94% sensitivity and accuracy of 95% (Nikiforova, Tseng, Steward, Diorio, & Nikiforov, 2008). In more recent study Rulong Shen established four microRNA expression panel (*microRNA - 146b; 221; 187* and *30d*) in training set of 60 FNAB that had histopathological diagnosis and tested it in 68 FNAB validation set (24 benign nodules and 44 malignant nodules - FVPTC=3; FTC=8; PTC=33). For the validation sample set they obtained a diagnostic accuracy of 85,3%, sensitivity of 88,9%, specificity of 78,3%, PPV of 89%, and NPV of 78%. For the 30 atypia cases in the validation sample set they obtained a diagnostic accuracy of 73,3%, sensitivity of 63,6%, specificity of 78,9%, PPV of 64%, and NPV of 79%, however the authors conclude that this mi-

croRNA expression panel is subject for inaccurate results in FTC in indeterminate FNAB cases (Shen et al., 2012). These two studies show that microRNA amplification in FNAB samples is feasible and that *microRNA - 146b; 221; 187* can be potentially used in microRNA expression panel to identify PTC in FNAB with indeterminate cytology. Although promising as diagnostic markers further studies that include many more tumor or indeterminate FNA samples as well as different combinations of microRNA are needed.

2.4 Protein based biomarkers

Protein-based assays probably may have most practical potential for clinical use than PCR-based assays since most clinical laboratories are familiar with immunocytochemistry (ICC) (Griffith, Chiu, Gown, Jones, & Wiseman, 2008). On the other hand, tremendous variability in specimen processing and interobserver interpretation of results often prevents easy and fast introduction of a protein marker into widespread clinical practice.

Till now the most studied immunohistochemistry panels include Galectin-3 (Gal-3), *HBME-1*, and *CK19* (Griffith, Chiu, Gown, Jones, & Wiseman, 2008; Saleh et al., 2009). Gal-3 encoded by *LGALS3* gene is a carbohydrate-binding lectin with an affinity for galactosides. It has diverse functions that include cell growth and cycle regulation, tumorigenesis, and apoptosis (Liu & Rabinovich, 2005). It is one of the most studied yet controversial markers in thyroid malignancy (Bartolazzi et al., 2008). One of the largest prospective multicenter studies was performed by the Italian Thyroid Cancer study group. Immunocytochemistry of Gal-3 in cellblocks of 544 patients with indeterminate cytology revealed sensitivity of 78% and specificity of 93%. However researchers concluded that although immunocytochemistry for Gal-3 could not replace FNA cytology, but it could serve as a complimentary method and/or be a part of a diagnostic panel (Bartolazzi et al., 2008). Hector Battifora mesothelial cell (*HBME*)-1 monoclonal antibody recognizes an antigen on the microvilli of mesothelioma cells (Sheibani, Esteban, Bailey, Battifora, & Weiss, 1992). The same antigen has also been identified in well differentiated thyroid cancer. In several recently published studies in FNA samples with indeterminate or suspicious cytology *HBME-1* immunocytochemistry had a sensitivity of 79–87% and a specificity of 83–96% (Raggio et al., 2010; Torregrossa et al., 2010). *CK19* has been found to be upregulated in malignant thyroid cells. Positive *CK19* staining is present in FNA samples from PTC and has been found to be useful in differentiating suspicious thyroid cytological specimens (Khurana, Truong, LiVolsi, & Baloch, 2003). Recent was published a study where *CK19* and *HBME-1* was tested in 150 FNAB with corresponding operation specimen for histopathological evaluation, by applying liquid based cytology. ICC results were defined as non-contributory, favoring benign, favoring malignant, or indeterminate. In 48 cases ICC was helpful for discriminating benign nodules from malignant with cytology of AUS, FN, and SM-PTC (42% of these lesions). Six (4%) ICC results were false positive (rather malignant than benign) but none were false negative (rather benign than malignant). Results for indeterminate cytological cases favored malignant or benign disease with sensitivity, specificity, NPV and PPV of 100%, 85,2%, 100%, and 86,2%, respectively. In conclusions authors proposed that immunocytochemistry of thyroid FNA with CK 19 and HBME1 antibodies can reduce the false-positive and false-negative results of single morphological analyses thus improving diagnostic accuracy and reducing the need for surgical controls (Cochand-Priollet et al., 2011).

2.5 Epigenetic biomarkers

An increasing body of evidence suggests that epigenetic changes play important roles in thyroid tumor genesis, as a result of their effects on tumor-cell differentiation and proliferation. Epigenetic alterations are changes around a gene that alter gene expression without affecting the nucleotide sequence of the gene and play a fundamental role in the regulation of human gene expression. Two epigenetic mechanisms are commonly used by cells to regulate gene expression - DNA methylation and histone modifications (Bird, 2002; Yoo & Jones, 2006). Epigenetic changes in tumorpromoting, tumor-suppressor and thyroid-specific genes contribute to the deregulation of thyrocyte growth and different aspects of tumorigenesis, such as apoptosis, motility and invasiveness (Xing et al., 2003). For example Xing with colleges showed that hypermethylation of *RAS* association domain family member 1 (*RASSF1)*, a known tumor suppressor gene, are found in 75% (9 of 12) of FTC as well as in a smaller percentage of benign adenomas (44%), and PTC (20%), these data indicates that this epigenetic silencing possibly occurs early in thyroid tumorigenesis (Xing et al., 2004).

In one of the most recent study Stephen and colleges examined aberrant promoter methylation in 24 tumor suppressor genes in 21 patient samples (11 - PTC, 2 - FTC, 5 normal thyroid and 3 hyperthyroid samples) by applying methylation specific multiplex ligation-dependent probe amplification (MS-MLPA) assay and in the *NIS* gene using methylation-specific PCR (MSP). The most frequently methylated genes were *CASP8* (17/21), *RASSF1* (16/21) and *NIS* (9/21), however these genes where methylated in the normal samples - 5/5, 4/5 and 1/5 respectively and in the hyperthyroid samples 3/3, 2/3 and 1/3 respectively. In the thyroid cancers *CASP8* was methylated in 9/13, *RASSF1* in 10/13 and *NIS* were methylated in 7/13. *CASP8, RASSF1* and *NIS* were also methylated in 3/11, 4/11 and 3/11 normal thyroid tissue matched with thyroid cancer cases. The authors conclude that aberrant methylation of *CASP8, RASSF1*, and *NIS* probably is an early change in thyroid tumorigenesis (Stephen et al., 2011). However further confirmation and validation of these findings in larger patient cohorts and FNA is needed to support these gene methylations as early makers of thyroid tumorigenesis.

2.6 Peripheral blood biomarkers

All of the studies to identify pre-operative molecular biomarkers described above have been based on material obtained from thyroid nodule FNA. Another way to diagnose malignant nodule preoperatively is to identify thyroid cancer specific molecular biomarkers in blood. Since thyroglobulin, no new blood tests for differentiated thyroid cancer have been introduced into routine clinical practice. A unique analysis of follicular thyroid nodules in 2004 has been described based on blood sample collection and measurement of circulating thyrotropin receptor (*TSHR*) mRNA which is derived from circulating thyroid cancer cells (Chinnappa et al., 2004). Recently there was published a study were *TSHR* mRNA was measured by qRT-PCR in blood drawn perioperatively in 526 patients undergoing thyroid surgery, postoperatively in 418 patients undergoing differentiated thyroid cancer follow-up and in 151 patients monitored for known benign nodule. In patients with malignant or suspicious cytology, preoperative evaluated *TSHR* mRNA had 96% of differentiated thyroid cancer diagnosed on histopathology, whereas 95% of patients with undetectable mRNA and benign thyroid sonography had benign nodules. Elevated *TSHR* mRNA levels became undetectable in all patients with differentiated thyroid cancer on the first postoperative day, except five patients who manifested persistent or recurrent cervical disease within the year. Authors report that utilizing a preoperative combination of diagnostic ultrasound, thyroid nodule FNA cytological diagnosis of follicular neoplasm and a *TSHR* mRNA diagnostic performance is sensitivity of 97%; specificity of 88

%; PPV of 88% and NPV of 95% for predicting thyroid cancer (Milas et al., 2010). However, the above publications have occurred at a single institution and an expanded clinical experience from other institutions is needed to verify this algorithmic approach.

In recent years several studies have demonstrated that miRNAs are released into the blood from cancer cells and exist there in a remarkably stable form thus suggesting that circulating miRNA may be used as minimal-invasive biomarkers (Chen et al., 2012; Mitchell et al., 2008). Since that, numerous studies have attempted to identify cell-free miRNAs with the diagnostic relevance in the blood from patients with different types of cancer. However, to the best of our knowledge, only one of these studies was focused on the thyroid cancer. Yu and colleges from China have determined serum miRNA expression profiles using Solexa sequencing followed by qRT-PCR validation in 245 samples (106 patients with PTC, 95 patients with benign nodules and 44 healthy controls). A panel of miRNA was evaluated in the sera of patients with PTC or benign nodules. This resulted in the identification of three microRNAs (*let-7e, miR-151-5p, and miR-222*) whose expression levels were significantly increased in the sera from PTC patients relative to patients with benign nodules and healthy donors. Serum *let-7e, miR-151-5p*, and *miR-222* levels correlated with certain clinicopathological variables, such as nodal status, tumor size, multifocal lesion status and metastasis. Furthermore, this study showed that expression of serum *miR-151-5p* and *miR-222* in a subset of PTC patients decreased significantly after tumor excision (Yu et al., 2012). Taken together, this study demonstrated the feasibility of detecting thyroid cancer-associated microRNAs in the blood of patients; however the diagnostic value of circulating microRNAs remains to be determined.

Study design, substrate and sample size	Method	Biomarkers	Diagnostic value/outcome	Reference
Prospective analysis of 967 consecutive thyroid FNA samples (AUS/FLUS - 653, FN/SFN - 247, SMC - 67) from 729 patients. Histologically confirmed was 513 FNA samples (AUS/FLUS - 32 malignant; 184 - benign; FN/SFN - 58 malignant; 152 - benign; SMC - 28 malignant; 23 benign)	PCR assay	BRAF V600E, NRAS, HRAS, KRAS, RET/PTC1, RET/PTC3, PAX8/PPAR γ	AUS/FLUS Sn - 63%, Sp - 99%, PPV - 88%, NPV - 94%; FN/SFN Sn - 57%, Sp - 97%; PPV - 87%; NPV - 86%; SMC Sn - 68%, Sp - 96%, PPV - 95%; NPV - 72%.	(Nikiforov et al., 2011)
Prospective analysis of 156 FNAB samples from patients undergoing thyroidectomy and results were compared with the definite histological outcome comprising 7 FTC, 11 PTC, 40 FA, 53 adenomatous nodules, 45 surrounding normal thyroid tissues	qRT-PCR	ADM3/TFF3 and ADM3/ACTB	Distinction of FA and malignant follicular neoplasia with NPV - 94% and PPV - 86%.	(Karger et al., 2012)
Multicenter prospective analysis of 4812 FNA (577 were indeterminate) from 3789 patients involving 49 clinical sites as reference using results of blinded histopathological review from 413 resected nodules with indeterminate cytology. After sample	Human exon microarrays	167 gene expression panel	Performance of the data set of indeterminate nodules where following: Sn - 92%; Sp - 52%; PPV - 47%; NPV - 93%.	(Alexander et al., 2012)

inclusion criteria were met, gene expression panel where used to test 265 indeterminate nodules				
Testing of 7 microRNA panel in 62 FNAB samples. Based on cytological analysis 13 patients had operation (4malignant, 8 atypical cytology) and histopathological analysis revealed 8 malignant (7 PTC and 1 FTC) nodules and 5 hyperplastic nodules	qRT-PCR	miR-187, miR-221, miR-222, miR-146b, miR-155, miR-224, and miR-197	Overall performance of the data set resulted in 100% specificity, 94% sensitivity and accuracy of 95%.	(Nikiforova et al., 2008)
Four microRNA expression panel that where established in training set of 60 FNAB that had histopathological diagnosis and analyzed in 68 FNAB validation set (24 benign nodules and 44 malignant nodules (FVPTC=3; FTC=8; PTC=33).	qRT-PCR	miRNA - 146b; 221; 187 and 30d	For the validation sample set authors obtained a diagnostic accuracy of 85,3%, sensitivity of 88.9%, specificity of 78,3%, PPV of 89%, and NPV of 78%. For the 30 atypia cases in the validation sample set they obtained a diagnostic accuracy of 73,3%, sensitivity of 63,6%, specificity of 78,9%, PPV of 64%, and NPV of 79%.	(Shen et al., 2012)
Multicenter, prospective protein expression analysis in 150 FNAB with corresponding operation specimen for histopathological evaluation, where 42 % was AUS, FN, and SM-PTC.	ICC	CK19 and HBME-1	Results for indeterminate cytological cases favored malignant or benign disease with sensitivity, specificity, NPV and PPV of 100%, 85,2%, 100%, and 86,2%, respectively.	(Cochand-Priollet et al., 2011)
Circulating thyrotropin receptor which is derived from circulating thyroid cancer cells was measured by qRT-PCR from blood drawn preoperatively in 526 patients undergoing thyroid surgery, postoperatively in 418 patients undergoing differentiated thyroid cancer follow-up and in 151 patients monitored for known benign nodule	qRT-PCR	TSHR mRNA	Combination of diagnostic ultrasound, thyroid nodule FNA cytological diagnosis of follicular neoplasm and a TSHR mRNA diagnostic performance was: sensitivity of 97%; specificity of 88 %; PPV of 88% and NPV of 95% for predicting thyroid cancer.	(Milas et al., 2010)
Promoter methylation of 24 tumor suppressor genes in 11 - PTC, 2 - FTC, 5 normal thyroid and 3 hyperthyroid samples	MS-MLPA assay and MSP for NIS gene	CASP8, RASSF1and NIS	In the normal samples, CASP8, RASSF1 and NIS were methylated in 5/5, 4/5 and 1/5 respectively. In the hyperthyroid samples, CASP8, RASSF1 and NIS were methylated in 3/3, 2/3 and 1/3 respectively. In the thyroid cancers,	(Stephen et al., 2011)

			CASP8, RASSF1, and NIS were methylated in 9/13, 10/13, and 7/13 respectively.	
3 identified in serum miRNA validation in 245 samples (106 patients with PTC, 95 patients with benign nodules and 44 healthy controls)	Serum miRNA identification by Solexa followed by qRT-PCR validation	let-7e, miR-151-5p, miR-222	AUC of 0,917 (Sn - 87,8% and Sp - 88,4%) and an AUC of 0,897 (Sn - 86,8% and Sp - 79,5%) in separation the PTC group from the benign and healthy control groups.	(Yu et al., 2012)

Table 1: Recent studies investigating new biomarkers for differential diagnostics of thyroid nodules.

3 Conclusions

In summary, until now there were no diagnostic tools that could help the clinicians in decision-making and management of cytological indeterminate thyroid nodules. Currently, the first auxiliary biomarkers assays that can improve FNA accuracy are being introduced into some clinics and the experience gained by their exploitation will be helpful for improving the guidelines for the management of thyroid nodules. In parallel, a number of novel biomarkers with remarkably high sensitivity and specificity have been identified, however they have to undergo thorough validation in prospective, multi-centre studies before considering for the exploitation in routine clinical practice. So far, fewer studies have succeeded in the identification of reliable biomarkers in patients' blood. However, the discovery of cancer-associated miRNAs in the blood of patients with thyroid cancer give rise to new hope that profiling microRNAs in the serum might lead to the identification of microRNA profiles that could be exploited for the development of non-invasive diagnostic assays. Currently, some molecular test panels are available which can aid in the decision making process for the management in cytological indeterminate FNAB cases. However in near future prospective, multi - canter validation studies of these and new developed biomarker tests should help to clinician in decision making and treatment management of cytological indeterminate thyroid nodules. But still the potential introduction of molecular markers as an adjunct to cytology diagnosis needs to take in account for inter- and intraobserver differences in cytological diagnoses as well as differences in inter- and intraobserver pathological evaluation.

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Abbreviations

3' UTR - three prime untranslated region

AACE - American Association of Clinical Endocrinologists
AC - anaplastic thyroid cancer
ACTB - actin, beta
ADM3 - adrenomedullin 3
ATA - American Thyroid Association
AUC - Area Under the Curve
AUS - Atypia of undetermined significance
BRAF - V-raf murine sarcoma viral oncogene homolog B1
CASP8 - caspase 8
CK19 - cytokeratin-19
EI - elasticity index
ETA - European Thyroid Association
FA - follicular adenoma
FAP - familial adenomatous polyposis
FLUS - follicular lesion of undetermined significance
FMTC - familial medullary carcinoma
FN/SFN - follicular neoplasm or suspicious for follicular neoplasm
FN1 - fibronectin 1
FNA – fine-needle aspiration FNAB
FTC- follicular thyroid cancer
FVPTC - follicular variant of papillary thyroid cancer
GALE - UDP-galactose-4-epimerase
HBME-1- Hector Battifora mesothelial cell - 1
ICC - immunocytochemistry
LGALS3 - lectin, galactoside-binding, soluble, 3
MAPK - mitogen-activated protein kinase
MEN syndromes – multiple endocrine neoplasia syndromes
MET - hepatocyte growth factor receptor
miRNA or miR - microRNA
mRNS - messengerRNA11
MS-MLPA - methylation specific multiplex ligation-dependent probe amplification
MSP - methylation-specific PCR
MTC- medullary thyroid cancer
NCI - The National Cancer Institute
NIS - sodium/iodide symporter
NPV – negative predictive value
PAX8 - paired box 8
PLAB - growth differentiation factor 15
PPARγ - peroxisome proliferatoractivated receptor γ
PPV – positive predictive value
PTC – papillary thyroid cancer
QPCT - glutaminyl-peptide cyclotransferase
qRT-PCR - quantitive reverse transcription polymerase chain reaction

RAS - Rat sarcoma
RASSF1 - RAS association domain family member 1
RET/PTC - Rearranged in Transformation/Papillary Thyroid Carcinomas
SMN - Suspicious malignancy of nodule
Sn - Sensitivity
Sp - Specificity
SWE - shear wave elastography
TFF3 - trefoil factor 3
TG - thyroglobulin
TSH - Serum thyrotropin measurement
TSH – thyroid stimulating hormone
TSHR - thyrotropin receptor
US- ultrasonography

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2 Materials and methods

2.1 Biological samples

2.1.1 Patient tissue specimens

Tissue specimens of thyroid nodules, thyroid and breast cancer and adjacent normal tissues were obtained from patients undergoing surgery in Latvian Oncology Centre and Pauls Stradins University Hospital during the period 2009-2011. Tissue samples were macroscopically dissected after total or partial thyroidectomy from thyroid cancer patients and mastectomy from breast cancer patients by histopathologist during the surgery and stored in RNALater® (Applied Biosystems, USA) at -20°C until processing. Tissue sections were evaluated by an experienced pathologist and the diagnosis was established according to standard histopathological criteria. The tissue specimens were collected after the patients' informed consent was obtained in accordance with the regulations of Ethics Committee of the Institute of Experimental and Clinical Medicine, University of Latvia.

2.1.2 Tissue microarrays (TMAs)

For immunohistochemical analysis, Thyroid diseased tissue array (A401, ISU ABXIS, Seoul, Korea) and Thyroid cancer tissue array (A210, ISU ABXIS, Seoul, Korea) where purchased. Thyroid cancer TMA compromising duplicates of 33 various thyroid nodule tissues (11 PTC, 5 FTC, 2 ATC, 5 MTC, 5 adenomatous hyperplasia and 5 FA) and 4 non neoplastic thyroid tissues, total 70 of 1.0 mm spots. Thyroid disease TMA comprising duplicates of 30 various diseased thyroid tissues (5 Graves' disease, 4 Granulomatous thyroiditis, 5 Hashimoto thyroiditis, 5 adenomatous hyperplasia, 6 Hurthle cell adenoma and 5 FA) and 4 non neoplastic thyroid tissues, a total of 64 1.0 mm spots.

2.1.3 Cell cultures

Human anaplastic thyroid carcinoma cell line 8305C and human mammary carcinoma cell line MCF7 were purchased from European Collection of Cell Cultures (ECACC, UK) and maintained according to the manufacturer's protocols. MCF7 cells are established from the pleural effusion of 69 years old Caucasian female suffering from a breast adenocarcinoma. Cells exhibit some features of differentiated mammary epithelium including estradiol synthesis and formation of ducts. Cells are both the estrogen receptors and progesterone receptor positive. 8305C cells are established from 67 year old female suffering from undifferentiated thyroid carcinoma. Pathologically the carcinoma tissue contained residual well differentiated components suggesting well differentiated to undifferentiated carcinoma progression. Cells contain a C:G to T:A transition at the first base of p53 gene codon 273. MCF7 cells were cultured in DMEM supplemented with GlutaMax (Gibco, Life Technologies, CA, USA), 10% FBS (Gibco, Life Technologies, CA, USA) and 1% NEAA (non essential amino acid solution) (Sigma - Aldrich, MO, USA). 8305C cells were cultured in DMEM supplemented with GlutaMax, 10% FBS and 1% NEAA.

2.1.4 Serum samples

Serum samples of 143 consecutive patients with thyroid nodules, 100 melanoma, 55 breast cancer, 14 non-small cell lung cancer (NSCLC) patients as well as 96 cancer-free healthy individuals were provided by the Genome Database of Latvian Population. Ninety of the thyroid patients were diagnosed with FA, 38 with PTC, 6 with MTC, 3 with ATC and 6 with FTC. The blood samples were collected in venous blood collection tubes with clot activator and gel for serum separation (Becton Dickinson, NJ, USA) and stored at +4°C until processing. The samples were centrifuged and aliquoted within 48 hours and stored at -80°C. Clinico-pathological characteristics of the study population are provided in Original paper III - Table 1. Study procedures were approved by the Central Committee of Medical Ethics of Latvia and the ethical committee of the University of Latvia, Institute of Experimental and Clinical Medicine. The blood samples were collected after the patients' informed consent was obtained.

2.2 Cell biology methods

2.2.1 Cell culture transfection with plasmid constructs.

To generate stable TFF3-expressing 8305C and MCF7 cells they were transfected with pIRESneo3 (Clontech Laboratories, CA, USA) containing human TFF3 cDNA (GeneBank accession number NM_003226). TFF3 was amplified by PCR from normal thyroid tissue cDNA using primers containing unique restriction site sequence (Table 2). The obtained PCR product was digested with FastDigest EcoRI and NheI (Fermentas, Thermo Fisher Scientific, MA, USA) and cloned into pIRESneo3 vector. The expression cassette of pIRESneo3 contains the human cytomegalovirus (CMV) major immediate early promoter/enhancer followed by a multiple cloning site (MCS) that precedes stop codons in all three reading frames, a synthetic intron known to enhance the stability of the mRNA, the internal ribosome entry site (IRES) of encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA, followed by the neomycin phosphotransferase (NPT II) gene, and the polyadenylation signal from SV40. After selection with G418 (Clontech Laboratories, CA, USA) (selective antibiotic for neomycin resistance gene), nearly all surviving colonies are stably expressing the TFF3 mRNA, because of the IRES structure, TFF3 and neomycin phosphotransferase are translated from one mRNA, thus decreasing the need to screen large numbers of colonies to find functional clones.

For transfection 1×10^5 of 8305C and MCF7 cells were seeded in 24-well plate and transfected with vector containing TFF3 and empty vector as a control using TurboFect transfection reagent (Fermentas, Thermo Fisher Scientific, MA, USA). After 8 weeks of selection with G418 stable transfectants were obtained. For MCF7 cells G418 was used in 800 μg/μl concentration, but for 8305C cells 500 μg/μl.

2.2.2 Cell functional tests

Cell functional tests were performed as described in the original paper II. Briefly, growth curve analysis was done by seeding MCF7 and 8305C cells transfected with TFF3 containing vector and empty control vector at density 0.5×10^4 cells per well in 24-well plates and cultured

for 10 days. Triplicate wells were trypsinized and the cells were counted using hemocytometer every 24 hours.

For estradiol stimulation experiments, the 8305C cells were seeded in serum free media at a density 1×10^5 cells per well in 24-well plates in triplicates and treated with 10pM or 1μM β-estradiol (Sigma Aldrich, St. Louis, MO) diluted in 96% ethanol according to manufacturer's protocol or 96% ethanol as a control for 72 hours.

For spheroid cultures, single-cell suspensions of 8305C-TFF3, 8305C-pIRES, MCF7-TFF3 and MCF7-pIRES cells were plated at a density 2×10^3 cells per ml in 96-well ultra-low attachment plates (Corning, NY, USA) in serum-free DMEM/F12 medium containing 1X B-27 supplement Minus AO, EGF (20ng/ml), bFGF (10ng/ml) and 1% methylcellulose (Sigma-Aldrich, St. Louis, MO, USA). After 7 days in culture, the tumour spheroids containing at least 16 cells were counted under an inverted microscope.

Cell proliferation was assessed using Click-iT® EdU assay (C10424; Life Technologies, CA, USA) according to the manufacturer's protocol. Briefly, the cells were seeded at density $5x10^5$ cells per well in 6-well plate in triplicates, grown until 80-90% confluence and incubated with 30 μM 5-ethynyl-2´-deoxyuridine (analog of timidine) for one hour. The cells were harvested and, after the fixation and permeabilization, stained with Alexa Fluor 647 azide. The DNA content was analyzed by flow cytometry on the BD FACSAria II cell sorter (BD Biosciences, CA, USA) using BD FACSDiva software (BD Biosciences, CA, USA).

2.3 RNA isolation and reverse transcription

Bead based tissue homogenisation was performed by using the FastPrep-24 homogenizer (MP biomedicals, USA) and Lysing Matrix D (MP biomedicals, USA) in 1 ml MirVana Lysis Buffer (Life Technologies, CA, USA). For thyroid tissues, the homogenisation protocol was three times for 40 seconds with 6 m/s speed, but for breast tissues one time for 40 seconds with 6 m/s speed. The supernatant of homogenized tissues was transferred to new tube and subjected to the extraction of total RNA according to manufacturer's protocol. Isolated RNA was further treated with DNase according to DNA Free kit protocol (Life Technologies, CA, USA). cDNA was synthesized by random hexamer priming from 4 μg of total RNA by using High Capacity cDNA synthesis kit (Life Technologies, CA, USA) according to manufacturer's protocol. Cells were directly lysed in 100 μl - 1 ml mirVana™ PARIS (Life Technologies, CA, USA) lysis buffer, depending on cell amount followed by RNA isolation according to manufacturer's protocol. Total RNA was treated with DNase and cDNA was synthesized from 500 ng to 4 μg of total RNA depending on the amount of cells in different experiments.

2.4 Expression analyses

2.4.1 mRNA expression analyses

Quantitative mRNA expression analyses were done as described in original paper I. RT-PCR (qPCR) reactions were performed using 2 μl of 1:10 diluted cDNA reaction mixtures, ABSolute Blue™ SYBR green Low ROX (Thermo Scientific, USA) on ABI7500 sequence detection system (Applied Biosystems, USA). Appropriate primer concentrations were established by cDNA 4 log serial dilution curves to ensure amplification efficiency over 95% and

the specificity of the amplification products were verified by the melting curve analysis. qPCR conditions were as follows: hold for 15 minutes at 95^0C, follow by two step PCR for 40 cycles of 95^0C for 15 seconds, followed by 60^0C for 1 minute. All reactions in tissues were performed in duplicates, but in cell cultures - in triplicates. To normalize the expression data in the sets of various normal or cancer tissues a normalisation factor (NF) was calculated for each cDNA from the expression values of most stable reference genes in each set determined among seven most often used housekeeping genes (*PGK1*, *PLA2*, *TUBA3*, *ACTB*, *GAPDH*, *TBP*, *POLR2A*) (Primer sequences are provided in Table 1) by using the open-access software GeNorm Win3.4 (http://medgen.ugent.be/~jvdesomp/genorm). The most stable genes were *POLR2A* and *PGK1* for thyroid tissues, *ACTB*, *PGK1* and *GAPDH* for breast tissues and *ACTB* and *POLR2A* for cell lines. Pooled cDNA from 10 thyroid tissue samples was used as a reference sample and included in each experiment to allow the comparison of TFF 3 expression in tissues across multiple plates. Expression level of each gene was determined relative to its expression in the sample with the highest expression (lowest Ct value). The analysis of relative gene expression (RQ) data was performed using the $2^{-\Delta CT}$ method and to eliminate variations of cDNA quantity and quality the data were normalised by using NF for each sample ($RQ=(2^{-\Delta CT})/NF$).

2.4.2 Protein expression analysis in tissues with immunohistochemistry (IHC)

The level of TFF3 protein expression was studied by IHC in various diseased thyroid tissues as described in original paper II. Briefly, immunohistochemical staining of thyroid disease and cancer tissues with TFF3 antibody (ab57752, monoclonal, Abcam, Cambridge, UK) was performed according to the AccuMax protocol. The tissue arrays where deparaffinised and rehydrated in xylene and ethanol followed by quenching of peroxidise. Epitope retrieval was performed by heating the slides in microwave for 15 min in 10mM sodium citrate buffer. Next, the tissue sections were incubated with anti-TFF3 antibody (1:300) overnight at 4 ^{o}C and the antibody binding was detected with anti-mouse IgG secondary antibody conjugated with peroxidase (A2554, Sigma - Aldrich, MO, USA) at 1:50 dilution. Colorimetric detection was performed with DAB (Sigma - Aldrich, MO, USA) followed by counterstaining with hematoxylin. Further, the slides where dehydrated, cleared with xylene and coverslips were mounted with Canadian Balsam (Sigma - Aldrich, MO, USA). and examined under light microscope (Leica DM3000, Germany).

2.4.3 Protein expression analysis by Western Blot

Protein expression analysis with Western blot was done as described in original paper II. Briefly, protein was isolated simultaneously with the total RNA from ~1×10^5 - 2×10^6 cells by using mirVana^{TM} PARIS kit according to the manufacturer's instructions, precipitated with isopropanol and dissolved in 100 µl 8mM Urea in 60 mM Tris-HCl and 1% SDS. Ten micrograms of total protein were applied per lane and separated by 10% SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes and stained with Ponceau S solution to ensure the normalisation control for protein loading. The membranes were destained, blocked with 5% (w/v) fat-free milk and then incubated with the following primary antibodies: TFF3 (ab57752, monoclonal, Abcam, Cambridge, UK) (1:1000), E-cadherin (sc8426, monoclonal, Santa Cruz Biotechnology, TX, USA) (1:1000) and β-actin (ab8224, monoclonal, Abcam, Seoul, Korea)

(1:2000). After washing, the membranes were incubated with peroxidise - conjugated goat anti-mouse secondary antibody (A2554, polyclonal, Sigma - Aldrich, MO, USA) (1:3000), washed and processed with ECL Select Western Blotting Detection Reagents (GE Healthcare, WI, USA) according to manufacturer's instructions.

2.5 Statistical analysis

Statistical analyses were done as described in original paper I and II. Briefly, a non-parametric Mann-Whitney *U* test was used to compare the RQ values of each candidate gene between two independent groups of tissue samples (benign vs malignant nodules; malignant nodules vs all normal tissue specimens). The receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) was calculated to evaluate the diagnostic performance of each marker gene and biomarker model. Youden index (J) was used to define cut-off points on the ROC curves with the maximal sum of sensitivity and specificity. Multivariate logistic regression was used to examine associations between thyroid nodule histopathological status and the gene expression. The initial multivariate logistic regression model included all individual genes and all combinations. Stepwise backward elimination based likelihood ratio test was used to drop out insignificant terms from the initial model. The predicted probability for each sample was calculated and used as input to generate ROC curve. As all samples were used for regression model generation the estimated AUC may be over optimised and to correct this bias leave one-out cross validation (LOOCV) was used to validate biomarkers and combinations of them to eliminate overestimated values.

For growth curve, proliferation test, β-estradiol and gene expression ratio (Primer sequences are provided in Table 2) experiments in cell cultures unpaired two tailed t test were performed. $P<0,05$ was considered as statistical significant. The statistical analyses were performed with SPSS 17.0 (SPSS, USA), Genex (Multid, Sweden) and GraphPadPrism 5 (GraphPad, USA).

2.6 Production and processing of phage displayed antigen microarrays

Production and processing of phage display antigen microarrays was done as described in original paper III. Briefly, for the production of 75-feature TAA microarray, a panel of 65 recombinant T7 phage clones previously selected from phage-displayed cDNA expression libraries and 10 non-recombinant phage clones was assembled and simultaneously amplified to high titre (~2-10×10^8 pfu/µl) in *E. coli* BLT 5616 cells using 96 well culture plates (Whatman, UK). The lysates were arrayed in duplicates onto nitrocellulose-coated 16-pad FAST slides (Maine Manufacturing, ME, USA) using a QArray Mini microarrayer (Genetix, UK). The slides were dried, blocked with 7% (w/vol) milk powder in TBS, 0.05% Tween 20, incubated with 80 µl of 1:200 diluted patients' sera that were preabsorbed with UV-inactivated *E.coli*- phage lysates, washed 4 times in TBS, 0.5% Tween 20 for 15 min, and then incubated with monoclonal anti-T7 tail fiber antibody (Novagen, SanDiego, CA, USA). Next, the microarrays were incubated with Cy5 labelled goat anti-human IgG antibody (1:1500) and Cy3 labelled goat anti-mouse IgG antibody (1:3000) (Jackson ImmunoResearch, PA, USA) for 1 h, then washed in TBS, 0.5% Tween 20, rinsed with distilled water and dried by centrifugation. A reference serum was included in each series of experiments. The arrays were scanned at 10 µm resolution in

PowerScanner (Tecan, Switzerland) with 532 and 635 nm lasers, the results were recorded as TIFF files and the data were extracted using GenePix software.

2.7 Microarray data processing and statistical analysis

The microarray data were processed and analyzed as described in original paper III using an *ad hoc* program composed in R language. Briefly, the mean Cy5 and Cy3 signals were background subtracted, averaged between replicates, and the Cy5/Cy3 ratios were calculated for each antigen. In order to eliminate variations introduced by the custom production of microarrays and variable background intensities of different sera, the data were median-centred and scaled across the slides. A cutoff value for defining sero-postive antigens in each field was defined as the mean signal intensities of all negative control spots (non-recombinant T7 phage) plus 3 standard deviations (SD). Fisher's exact test was applied to determine the level of significance for the binominal frequency data.

Then the rank (R) for each antigen was calculated, taking into account the signal intensity (I) and frequency of reactivity (N) with sera from cancer patients (Ca) compared to healthy control sera, using the following formula:

$$R_i = \left(\sum \frac{I_{Cai}}{N_{Cai}}\right) - 2\left(\sum \frac{I_{HDi}}{N_{HDi}}\right)$$

Coefficient 2 was introduced in this formula in order to decrease the rank of antigens reacting with sera from healthy controls. Finally, a score (S) for each serum was calculated by summing up the intensities of sero-postive antigens as follows: $S = \sum_{i=1}^{n} \sqrt{R_i} \times I_i$. The non-parametric Mann-Whitney U test was used to compare the serum scores between two independent groups of samples. The receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) was calculated to evaluate the diagnostic performance of the serum scores.

Table 1. Primers used for the RT-qPCR

GENE	FORWARD PRIMER 5' - 3'	REVERSE PRIMER 5' - 3'
ACTB	AATCTCATCTTGTTTTCTGCGC	AGTGTGACGTGGACATCCG
BIRC5	CAGCCCTTTCTCAAGGACC	AAGCAGAAGAAACACTGGGC
CCND1	TGGTGAACAAGCTCAAGTGG	ATCACTCTGGAGAGGAAGCG
CCNE1	ATCAGCACTTTCTTGAGCAACA	TTGTGCCAAGTAAAAGGTCTCC
CDH1	AGAAACAGGATGGCTGAAGG	AGCACCTTCCATGACAGACC
CDK1	TCGAAAGCCAAGATAAGCAAC	CCGCAACAGGGAAGAACAGT
CDK2	GCTAGCAGACTTTGGACTAGCCAG	AGCTCGGTACCACAGGGTCA
CDK4	CTGGTGTTTGAGCATGTAGACC	AAACTGGCGCATCAGATCCTT
CDKN1B	CTGCAACCGACGATTCTTCTACT	GGGCGTCTGCTCCACAGA
CITED1	GCTCTGAAATGCCAACAACG	TGGTTCCATTTGAGGCTACC
DPP4	TGATGCTACAGCTGACAGTCG	CTGAGCTGTTTCCATATTCAGC
ESR1	AGGATCTCTAGCCAGGCACA	AAGCTTCGATGATGGGCTTA
ESR2	ACCAAAGCATCGGTCACG	CATGATCCTGCTCAATTCCA
FN1	CCCATCAGCAGGAACACCTT	GGCTCACTGCAAAGACTTTGAA
FOXE1	CTCCCCATTCCCACAAAAACC	CGGAGTTTTCGGACTTTTCAGG
GAPDH	GGGTCTTACTCCTTGGAGGC	GTCATCCCTGAGCTAGACGG
LGALS3	CTGATTGTGCCTTATAACCTGC	AAGCAATTCTGTTTGCATTGG
MET	TCTGCCTGCAATCTACAAGG	AAGGTGCAGCTCTCATTTCC
SLC5A5	ACAGCACAGTGGTCAGCGT	CTCTCCTCCCTGCTAACGAC
NKX21	GAATCTTTAAGCAGAGAA	TGAAGTTTGGTCTTTAGAGTC
PAX8	AAGTCCAGCATTGCGGCACA	GAGGGAAGTGCTTATGGTCC
PGK1	CTTAAGGTGCTCAACAACATGG	ACAGGCAAGGTAATCTTCACAC
PLA2	CCTGCATGAAGTCTGTAACTGAG	GACCTACGGGCTCCTACAAC
POLR2A	GGGTCATCTTCCCAACTGGAG	CACCAGCTTCTTGCTCAATTCC
SLC26A4	GGAACTGCAGCTAGTAGGGC	CCCAAATACCGAGTCAAGGA
TAZ	GTCACCAACAGTAGCTCAGATC	AGTGATTACAGCCAGGTTAGAAAG
TBP	CCACTCACAGACTCTCACAAC	CTGCGGTACAATCCCAGAAC
TFF3	GTACGTGGGCCTGTCTGC	GATCCTGGAGTCAAAGCAGC
TG	CAACTGACCTCCTTTGCCA	CACCAACTCCCAACTTTTCC
THRB	CAACCAGAAGGAAATCGCA	AAAGAGACCTCCTGCTCCG
TPO	GCAGTGTGGATTTAGTGCCA	ACTTGGATCTCCATGTCGCT
TSHR	AGCTGCTGCAGAGTCACATC	GATATTCAACGCATCCCCAG
TUBA3	TATGGCAAGAAGTCCAAGCTG	TACCATGAAGGCACAATCAGAG

3. Results

The results are presented here as original publications. The author's contribution to the enclosed publications are presented.

Review paper (presented in literature overview)
A. Ābols, K. Ducena, A. Linē, V. Pīrāgs. Relevance of molecular biomarkers for differential diagnostics of thyroid cancer. Breast, Cervical and Prostate Cancer (BK039-1-1). Paper ID 12090423294242
Contribution: Wrote partly the manuscript and prepared the tabular information.

Original Paper I (presented in results)
Ducena K, **Ābols A**, Vilmanis J, Narbuts Z, Tārs J, Andrējeva D, Linē A, Pīrāgs V. Validity of multiplex biomarker model of 6 genes for the differential diagnosis of thyroid nodules. *Thyroid Res.* 2011 Jun 27;4(1):11.
Contribution: Performed experimental work and data analysis, prepared graphical and tabular information and partly prepared manuscript.

Original Paper II (presented in results)
Artūrs Ābols, Kristīne Ducena, Diāna Andrejeva , Lilite Sadovska, Elīna Zandberga, Jānis Vilmanis, Zenons Narbuts, Juris Tārs, Jānis Eglītis, Valdis Pīrāgs and Aija Linē. Trefoil Factor 3 is Required for Differentiation of Thyroid Follicular Cells and Acts as a Context-dependent Tumor Suppressor. (manuscript accepted in Neoplasma).
Contribution: Performed experimental work, data analysis, prepared graphical and tabular information, wrote partly the manuscript.

Original Paper III (presented in results)
A. Ābols, K. Ducena, P. Zayakin, K. Siliņa, Z. Kalniņa, L. Sadovska, J. Tārs, J. Vilmanis, Z. Narbuts, J. Eglītis, V. Pīrāgs and A. Linē. Survey of autoantibody responses against tumor-associated antigens in thyroid cancer. Cancer Biomarker. 2014;14(5):361-9
Contribution: Partly performed the experimental work and data analysis, wrote partly the manuscript.

3.1 Biomarker identification of thyroid cancer tissues for development of pre - operative diagnostic test

Ducena et al. Thyroid Research 2011, 4:11
http://www.thyroidresearchjournal.com/content/4/1/11

RESEARCH Open Access

Validity of multiplex biomarker model of 6 genes for the differential diagnosis of thyroid nodules

Kristine Ducena[1*], Arturs Ābols[1,2], Janis Vilmanis[3], Zenons Narbuts[3], Juris Tārs[4], Diana Andrējeva[2], Aija Linē[2] and Valdis Pīrāgs[1,3*]

Abstract

Background: Currently the cytological examination of fine needle aspiration (FNA) biopsies is the standard technique for the pre-operative differential diagnosis of thyroid nodules. However, the results may be non-informative in ~20% of cases due to an inadequate sampling and the lack of highly specific, measurable cytological criteria, therefore ancillary biomarkers that could aid in these cases are clearly needed. The aim of our study was to evaluate the mRNA expression levels of 8 candidate marker genes as the diagnostic biomarkers for the discrimination of benign and malignant thyroid nodules and to find a combination of biomarkers with the highest diagnostic value.

Materials and methods: mRNA expression levels of eight candidate marker genes - *BIRC5, CCND1, CDH1, CITED1, DPP4, LGALS3, MET* and *TFF3* was measured by real-time RT-PCR in paired nodular and surrounding normal thyroid tissue specimens of 105 consecutive patients undergoing thyroid surgery and compared between different types of thyroid lesions.

Results: Significant differences in the mRNA expression levels between the normal and malignant thyroid tissues and between benign and malignant nodules were found for *BIRC5, CCND1, CITED1, DPP4, LGALS3, MET* and *TFF3*, but not *CDH1*. On a single gene basis, relative quantity (RQ) of *LGALS3* had the highest diagnostic value for the discrimination of malignant and benign thyroid nodules (AUC = 0.832, P < 0.0001 and 90.9% sensitivity and 65.6% specificity at the optimal cut-off on ROC curve). The only two-marker set that outperformed *LGALS3* was RQ sum of *LGALS3* and *BIRC5* (AUC = 0.841, P < 0.0001). An application of multivariate logistic regression analysis resulted in the generation of a multiplex biomarker model based on *LGALS3, BIRC5, TFF3, CCND1, MET* and *CITED1* that had considerably higher specificity than a single marker or two marker gene-based models (AUC = 0.895, P < 0.0001, 70.5% sensitivity and 93.4% specificity).

Conclusions: This study confirmed that mRNA expression levels of 7 out of 8 candidate genes analysed have a diagnostic value for the distinction of benign and malignant thyroid nodules. The multiplex biomarker model based on 6 genes outperformed a single marker or two marker-based models and warrants feasibility studies on FNA biopsies and the validation in a larger cohort of patients.

Background

Thyroid nodules are a very common clinical finding - the prevalence of palpable nodules ranges from 3 to 7% in the general population but can be as high as 50% based on ultrasonography or autopsy data [1]. Although only less than 5% of the palpable nodules are malignant lesions, thyroid cancers are the most common malignancy of endocrine organs. According to European Cancer Observatory data age-standardised incidence rate was 3.1 cases and 8.8 cases per 100 000 in men and women, respectively in Europe in 2008 and its incidence rates have steadily increased over the recent decades [2,3]. More than 95% of malignant lesions are derived from thyroid follicular cells and are divided into papillary and follicular carcinomas that differ mainly in the mode of metastatic spread (lymphatic and haematogenous spread, respectively) yet both are relatively indolent tumours with 5-year survival rates > 90%, and

* Correspondence: kducena@inbox.lv; pirags@latnet.lv
[1]Faculty of Medicine, University of Latvia, Raina Bulvaris 19, Riga, LV1586, Latvia
Full list of author information is available at the end of the article

undifferentiated or anaplastic carcinoma that is a highly aggressive and lethal cancer with 5-year survival rate below 1-17% [4]. A minority of thyroid carcinomas are derived from parafollicular cells (or C-cells) and are referred to as medullary carcinomas with 5-year survival rate of ~80% [4]. Benign nodules include hyperplasic follicular adenomas, multinodular goiter, thyroiditis and benign cysts [1].

The differential diagnosis of nodular thyroid disease is based on cytological examination of fine needle aspiration (FNA) biopsies, however the results may be non-informative in ~20% of cases due to an inadequate sampling and the lack of highly specific, measurable cytological criteria [4,5]. Furthermore, the principal diagnostic feature for follicular thyroid carcinomas (FTC) is capsular or vascular invasion and therefore it can not be reliably distinguished from follicular adenomas by analysis of FNA smears and the definite diagnosis relies on the histological examination of the postoperative surgical specimens [6]. Hence molecular biomarkers of malignancy that could reliably discriminate between malignant and benign nodules in the grey zone of thyroid FNA and classify tumours into the histological subtypes are clearly needed.

Gene expression profiling using cDNA microarrays or serial analysis of gene expression (SAGE) has revealed several hundreds of genes that are differentially expressed between malignant and benign thyroid nodules [7-9]. A number of them, including *LGALS3, MET* etc have been shown to be functionally involved in the carcinogenic process, have been validated by qRT-PCR and confirmed at protein level by immunohistochemistry [10-12]. However, several studies have demonstrated that none of these genes individually has sufficient sensitivity and specificity to be exploited as an independent diagnostic biomarker [13,14] while using very large panels of genes may not be practical. Therefore the definition of minimal set of marker genes that allows the correct classification of nodules is required in order to develop a clinically applicable biomarker assay. In the current study, we evaluated the diagnostic value of 8 candidate marker genes - *BIRC5, CCND1, CDH1, CITED1, DPP4, LGALS3, MET* and *TFF3* by analysing their mRNA expression levels in nodular and adjacent relatively normal thyroid tissue specimens of 105 consecutive patients with thyroid nodules who underwent thyroidectomy, and developed a multiplex biomarker model for the classification of the nodules.

Methods

Patients and tissue specimens

Paired specimens of thyroid nodules and surrounding normal tissues were collected from 105 consecutive patients undergoing total or partial thyroidectomy at the Latvian Centre of Oncology and Pauls Stradiņš Clinical University Hospital during the period 2009-2010. Tissue samples were macroscopically dissected by histopathologist during the surgery and stored in RNALater® (Applied Biosystems, USA) at -20°C until processing. Tissue sections were evaluated by experienced pathologist and the diagnosis was established according to standard histopathological criteria. Sixty one of the nodules were diagnosed as follicular adenomas (FA), 33 as papillary thyroid carcinomas (PTC), 5 as medullar thyroid carcinomas (MTC), 3 as anaplastic thyroid carcinomas (ATC) and 3 as follicular thyroid carcinomas (FTC). Selected clinical-pathological data of the patient groups are provided in Table 1. The specimens were collected after the patients' informed consent was obtained in accordance with the regulations of Ethics Committee of the University of Latvia Institute of Experimental and Clinical Medicine.

Table 1 Clinical and pathological characteristics of the patient groups

Characteristics	Thyroid cancer n = 44	Benign nodule n = 61
Gender		
Male	9	4
Female	35	57
Age at diagnosis		
Mean ± SD	55 ± 17	54 ± 15
Median (range)	55 (24-83)	57 (25-83)
Family history of cancer	37	49
TSH level μIU/mL		
Mean ± SD	2,31 ± 1,41	0,87 ± 0,74
Median (range)	1,32 (1,1-4,34)	0,75 (0,004-2,43)
US criteria		
Hipoechogenic nodule	9	14
No Halozones	0	1
Irregular frontier (non-homogenic)	6	14
Microcalcinates	4	9
Nodules > 3 cm	5	19
Central vascularization	3	3
Retrosternal	1	7
Swollen lymph nodes	2	0
No data	18	8
Histopathology		
PTC	33	
FTC	3	
MTC	5	
ATC	3	
FA		61

RNA extraction and cDNA synthesis

Tissue specimens were homogenised using the FastPrep-24 instrument and Lysing Matrix D (MP Biomedicals, USA) in 1 ml of Lysis solution® (Ambion, USA) followed by the extraction of total RNA using MirVana® (Ambion, USA) according to manufacturer's instructions. RNA extracted from tissue material was treated with DNAse prior to cDNA synthesis (Ambion, USA) and quantified by NanoDrop ND-100 spectrophotometer. cDNA was synthesized by random hexamer priming from 4 μg of total RNA by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer's instructions.

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) reactions were performed using 2 μl of 1:10 diluted cDNA reaction mixtures, ABSolute Blue™ SYBR green Low ROX (Thermo Scientific, USA) on ABI7500 sequence detection system (Applied Biosystems, USA). Appropriate primer concentrations were established by cDNA 4 log serial dilution curves to ensure amplification efficiency over 95% and the specificity of the amplification products were verified by the melting curve analysis. Sequences of primers used in this study are provided in Table 2. qPCR conditions were as follows: hold for 15 minutes at 95°C, follow by two step PCR for 40 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute. All reactions were performed in duplicates. To choose the appropriate reference genes, a set of seven candidate reference genes (*PGK1, PLA2, TUBA3, ACTB, GAPDH, TBP, POLR2A*) was tested for their mRNA expression stability in 10 thyroid tissue samples from 5 patients (2 PTC, 3 FA, 5 normal) and the two most stable genes (*POLR2A and PGK1*) were determined by using the open-access software GeNorm Win3.4 (http://medgen.ugent.be/~jvdesomp/genorm). Normalisation factor (NF) for each cDNA sample was then calculated by using GeNorm Win3.4 based on *PGK1* and *POLR2A* expression level. Pooled cDNA from 10 thyroid tissue samples was used as a reference sample and included in each experiment to allow the comparison across multiple plates. The expression level of each gene was determined relative to its expression in the sample with the highest expression (lowest Ct value). The analysis of relative gene expression (RQ) data was performed using the $2^{-\Delta CT}$ method and to eliminate variations of cDNA quantity and quality the data were normalised by using NF for each sample (RQ = $(2^{-\Delta CT})$/NF).

Statistical analysis

A non-parametric Mann-Whitney *U* test was used to compare the RQ values of each candidate gene between

Table 2 Primers used for qPCR

Name of the gene	Sequence (5'- > 3')	Size of PCR product (bp)	GenBank accession no.
Candidate genes			
LGALS3 F	CTGATTGTGCCTTATAACCTGC	100	NM_002306.3
LGALS3 R	AAGCAATTCTGTTTGCATTGG		
TFF3 F	GTACGTGGGCCTGTCTGC	121	NM_003226.3
TFF3 R	GATCCTGGAGTCAAAGCAGC		
DPP4 F	TGATGCTACAGCTGACAGTCG	164	NM_001935.3
DPP4 R	CTGAGCTGTTTCCATATTCAGC		
CITED1 F	GCTCTGAAATGCCAACAACG	174	NM_001144886.1
CITED1 R	TGGTTCCATTTGAGGCTACC		
MET F	TCTGCCTGCAATCTACAAGG	153	NM_001127500.1
MET R	AAGGTGCAGCTCTCATTTCC		
CDH1 F	AGAAACAGGATGGCTGAAGG	199	NM_004360.3
CDH1 R	AGCACCTTCCATGACAGACC		
CCND1 F	TGGTGAACAAGCTCAAGTGG	280	NM_053056.2
CCND1 R	ATCACTCTGGAGAGGAAGCG		
BIRC5 F	CAGCCCTTTCTCAAGGACC	152	NM_001168.2
BIRC5 R	AAGCAGAAGAAACACTGGGC		
Hausekeeping genes			
PGK1 F	CTTAAGGTGCTCAACAACATGG	119	NM_000291.3
PGK1 R	ACAGGCAAGGTAATCTTCACAC		
POLR2A F	GGGTCATCTTCCCAACTGGAG	164	NM_000937.4
POLR2A R	CACCAGCTTCTTGCTCAATTCC		

F - forward primer, R- reverse primer

two independent groups of samples (benign vs malignant nodules; malignant nodules vs all normal tissue specimens). The statistical significance was set at $p < 0.05$. The receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) was calculated to evaluate the diagnostic performance of each marker gene and biomarker model. Youden index (J) was used to define cut-off points on the ROC curves with the maximal sum of sensitivity and specificity. Multivariate logistic regression was used to examine associations between thyroid nodule histopathological status and the gene expression data as described by Laxman B et al [15]. The initial multivariate logistic regression model included all individual genes and all combinations. Stepwise backward elimination based likelihood ratio test was used to drop out insignificant terms from the initial model. The predicted probability for each sample was calculated and used as input to generate ROC curve. Leave one-out cross validation (LOOCV) as described earlier was used to validate biomarkers and combinations of them to eliminate overestimated values [15]. The statistical analyses were performed with SPSS 17.0 (SPSS, USA), Genex (Multid, Sweden) and GraphPadPrism 5 (GraphPad, USA).

Results and discussion

Selection of candidate marker genes

The selection of candidate marker genes was based on the functional involvement in various pathways contributing to the acquisition of malignant phenotype and/or previously reported differential expression in the malignant and benign thyroid nodules. Cyclin D1 encoded by *CCND1* is a crucial cell cycle regulator frequently upregulated at protein level in PTC [16]. *CITED1* encodes a cell cycle-dependent transcriptional cofactor involved in TGF-beta and Bmp signalling that may coordinate cellular differentiation and survival signals [17,18] and has been found to be overexpressed in PTC by expression profiling using cDNA microarrays [19], later confirmed as useful marker for FTC [6] and validated at protein level by IHC [20]. Loss of expression or function of E-cadherin - a cell-cell adhesion glycoprotein encoded by *CDH1*, has been demonstrated to contribute to the progression of various cancers, including poorly differentiated thyroid cancers by increasing proliferation, invasion and metastasis [21,22]. Overexpression of dipeptidyl-peptidase 4 encoded by *DPP4* in PTC has been demonstrated at protein level by several groups [23-25], however the mechanism how it may contribute to the development or progression of thyroid malignancies remains unknown. Survivin, an inhibitor of apoptosis encoded by *BIRC5*, has been shown to be overexpressed in a variety of cancers, including thyroid cancer, where it contributes to uncontrolled cancer cell growth and drug resistance [26,27]. Increased expression at the mRNA and protein level of the receptor for hepatocyte growth factor encoded by *MET* has been frequently observed in PTC, follicular variant of PTC and at lesser degree in FTC where it promotes tumour progression by facilitating cell proliferation, survival, migration, invasion, and metastasis [28-30]. However, to our knowledge, so far the diagnostic value of *CDH1*, *DPP4*, *BIRC5* and *MET* mRNA levels has not been established. Galectin 3 encoded by *LGALS3* plays an important role in cell-to-cell adhesion, cell-to-matrix interactions and the regulation of apoptosis and proliferation, and its overexpression correlates with thyroid cancer aggressiveness and metastasis [31,32]. Although *TFF3* mRNA has been shown to be overexpressed in several solid cancers, such as hepatocellular carcinoma, colon and prostate cancer, and suggested to contribute the malignant behaviour and metastasis [33-36], SAGE analysis of thyroid follicular adenomas and carcinomas demonstrated that it is downregulated in FTC [37] and later this finding was confirmed in PTC and ATC by other researchers [14,38], thus validating *TFF3* downregulation as a universal marker of cancers derived from thyroid follicular cells. It encodes a small secreted protein - trefoil factor 3, which is abundantly expressed at mucosal surfaces and promotes regeneration and repair. Interestingly, recent reports demonstrate that both galectin-3 and TFF3 are involved in ciliogenesis, epithelial cell differentiation and polarity [39,40], thus suggesting a yet unexplored role of the deregulation of these processes in the development of thyroid cancer.

mRNA expression analysis and the diagnostic performance of individual marker genes

mRNA expression analysis of the selected marker genes by RT-qPCR revealed statistically significant differences both between the normal and malignant thyroid tissues and between benign and malignant nodules for all genes except *CDH1*, thus further supporting their role the development and/or progression of thyroid cancer. *LGALS3, DPP4, MET, CITED1, CCND1* and *BIRC5* were found to be significantly upregulated while *TFF3* was downregulated in the malignant tissues (Table 3 and Figure 1). At first, all the genes were tested by ROC curve analysis as individual biomarkers, which demonstrated that *LGALS3* have the highest value for discriminating malignant from benign nodules in our sample set ($AUC = 0.832$, $P < 0.0001$). The cut-off of *LGALS3* expression level (RQ) that discriminates benign and malignant nodules with the highest accuracy (sensitivity 90.9%, specificity 65.6%) was determined to be 0.019. The sensitivity for the detection of various subtypes of

Table 3 Relative expression values and the diagnostic performance of the marker genes

Gene, protein name	RQ values - Mean ± SEM			Mann-Whitney U test - p-values		ROC curve - AUC (Asimptotic significance)	
	Normal	Benign	Malignant	Normal vs Malignant	Benign vs Malignant	Normal vs Malignant	Benign vs Malignant
LGALS3, Galectin-3	0.068 ± 0.013	0.035 ± 0.007	0.248 ± 0.067	2.2×10^{-5}	1×10^{-8}	0.715 (< 0.0001)	0.832 (< 0.0001)
TFF3, trefoil factor 3	0.115 ± 0.011	0.066 ± 0.007	0.037 ± 0.008	4.1×10^{-8}	0.0003	0.782 (< 0.0001)	0.696 (0.001)
DPP4, Dipeptidyl peptidase-4	0.011 ± 0.002	0.0146 ± 0.003	0.066 ± 0.016	0.0001	0.0004	0.693 (< 0.0001)	0.695 (0.001)
MET, hepatocyte growth factor receptor	0.048 ± 0.005	0.033 ± 0.004	0.110 ± 0.021	0.01	0.0005	0.610 (0.037)	0.689 (0.001)
CITED1, Cbp/p300-interacting transactivator 1	0.010 ± 0.002	0.021 ± 0.008	0.161 ± 0.048	0.001	0.001	0.654 (0.003)	0.67 (0.003)
CCND1, Cyclin D1	0.037 ± 0.004	0.036 ± 0.007	0.084 ± 0.017	0.002	0.001	0.647 (0.005)	0.67 (0.003)
BIRC5, Survivin	0.025 ± 0.011	0.009 ± 0.002	0.045 ± 0.018	0.0001	0.001	0.694 (< 0.0001)	0.668 (0.003)
CDH1,E-cadherin	0.171 ± 0.013	0.117 ± 0.009	0.158 ± 0.018	0.2	0.06	0.464 (0.497)	0.525 (0.697)

thyroid cancers was mutually comparable (Figure 2C) - 29 out of 33 PTC, 4 of 5 MTC and all FTC and ATC specimens were classified correctly thus confirming that *LGALS3* may serve as a universal diagnostic marker of the thyroid malignancies. However, 22 of 61 benign nodules were misclassified using this cut-off. As the ROC curve was constructed using all the samples, it may lead to overestimation of the AUC, therefore we next used LOOCV to validate it. The AUC for LOOCV *LGALS3* model dropped to 0.783, nevertheless it still outperformed the other marker genes. Although previously the diagnostic utility of *LGALS3* mRNA expression level has been questioned, our data show that the diagnostic accuracy of mRNA quantification is comparable to that reported in immunohistochemical studies [32]. The next marker genes with the highest diagnostic value in our sample set were *TFF3* (AUC = 0.696, P = 0.001) and DPP4 (AUC = 0.695 and P = 0.001). The optimal RQ cut-off for *TFF3* was determined to be 0.011 allowing the discrimination between benign and malignant nodules with sensitivity 54.6% and specificity 90.2%, while for *DPP4* the cut-off was 0.027 with sensitivity 45.6% and specificity 88.5%.

Interestingly, in 3 PTC and 2 MTC cases none or only one of the genes analysed were differentially expressed between cancerous and relatively normal tissues in specimens and they were consistently misclassified as benign nodules. These specimens are likely to represent a distinct molecular subtype of thyroid cancer, in which different molecular pathways predominate and therefore next these samples will be subjected to gene expression profiling using cDNA microarrays.

Development of multiplex biomarker model

To determine if a combination of markers could outperform the single biomarkers we systematically searched for two-marker ratios or two-marker sums, and determined their diagnostic performance by ROC curve analysis. In total, 27 two-marker combinations were evaluated, however only one of them - *LGALS3* and *BIRC5* RQ sum showed higher AUC for discriminating benign and malignant nodules than the best individual marker gene (AUC = 0.841, P < 0.0001 and AUC = 0.809, P < 0.0001 for the LOOCV model) (Figure 2A,B). At the best cut-off, the sensitivity was 79.5% and specificity was 78.7%. As shown in Figure 2D, this two-gene combination could detect all FTC and ATC cases but failed to detect 7 of 33 PTCs (21%) and 2 of 5 MTCs (40%) thus suggesting it may have lower value for diagnosing MTC, however this finding should be verified in larger cohort of patients. So far, the best reported two-marker set is *TFF3/LGALS3* ratio that has been shown to discriminate follicular carcinomas from follicular adenomas with 72.4% sensitivity and 83.3% specificity (or 80% and 91.5%, when the pathologically questionable cases were excluded) [41]. In our sample set it showed similar performance (AUC = 0.758, P < 0.0001, sensitivity 72.7%, specificity 85.3%). However, although it could improve the specificity, the overall accuracy of *TFF3/LGALS3* ratio was lower than that of *LGALS3* alone. Moreover, as *TFF3* is downregulated in cancer cells, the development of assay that is based on the measurement of its expression level in FNA biopsies consisting of a mixture of different cell types is technically more challenging than the assay

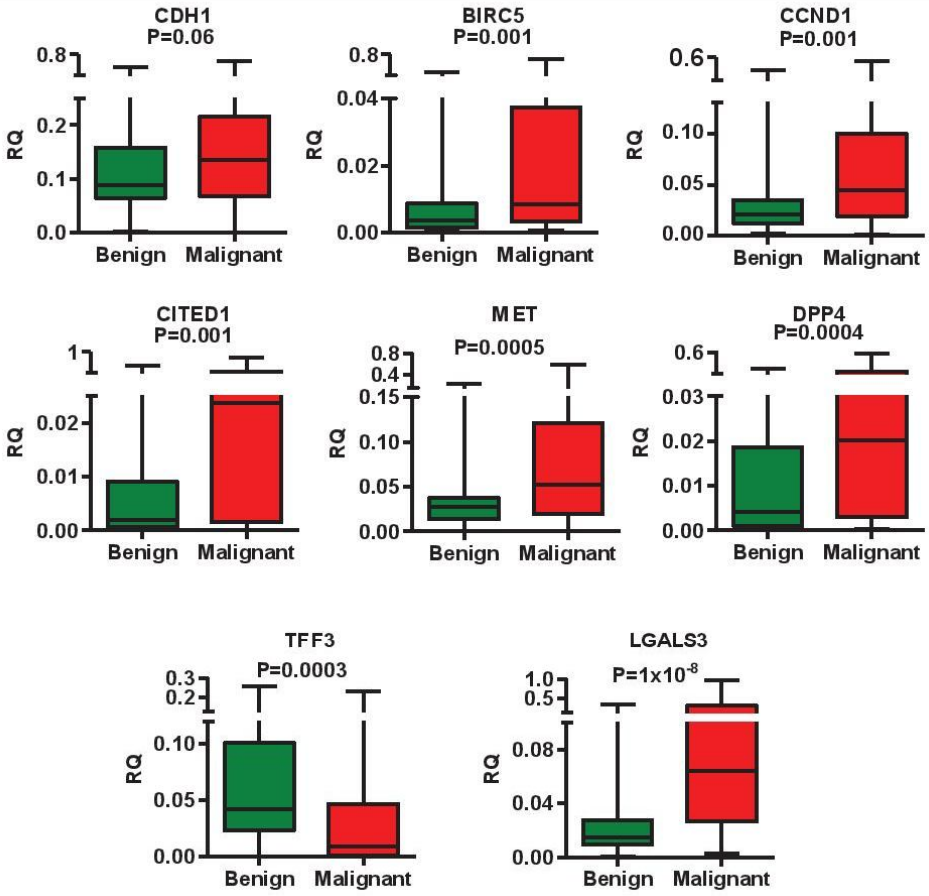
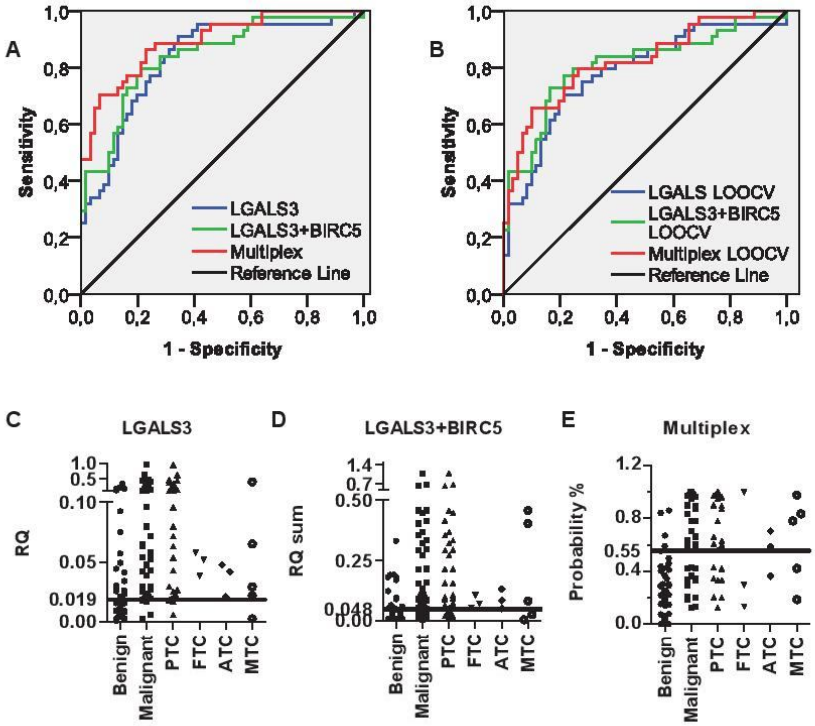


Figure 1 Median mRNA expression levels of the candidate marker genes in groups of 61 benign and 44 malignant thyroid nodules represented by box plots. The relative quantity (RQ) of each marker gene in each sample was calculated using $2^{-\Delta CT}$ method and normalised by using normalisation factor obtained by analysis of the two most stable reference genes (RQ = $(2^{-\Delta CT})$/NF). Boxes represent the 25th and 75th percentiles; whiskers represent the minimum and maximum values. Statistical significance was calculated using Mann-Whitney *U* test.

based on overexpressed genes, therefore *LGALS3* and *BIRC5* RQ sum seems to be more suitable for the clinical application.

Next, we applied a multivariate logistic regression analysis to define a multiple marker set that could improve the diagnostic performance over single markers or two-marker combinations. Similar approach has been previously successfully used by Laxman B et al to develop a multiplex biomarker model for the detection of prostate cancer [15]. This resulted in the multiplex model that included LGALS3 and BIRC5 RQ sum, TFF3 and LGALS3 RQ ratio, TFF3 and CCND1 RQ ratio, ratio between TFF3 RQ and MET, CITED1 RQ sum, ratio between TFF3 RQ and MET, BIRC5 RQ sum and ratio between TFF3 RQ and CCND1, BIRC5 RQ sum. Although the overall performance of the model (AUC = 0.895, P < 0.0001 and AUC = 0.823, P < 0.0001 for the LOOCV model) was only slightly improved over *LGALS3* alone or *LGALS3* and *BIRC5* RQ sum, at the best cut-off, this model shows 70.5% sensitivity and 93.4% specificity and as shown in Figure 2A it has considerably higher specificity that is a clear requirement for the development of clinically applicable biomarker assay.



Test Result Variable(s)	Area	Std. Error	Asymptotic Sig.	Sens.	Spec.	Asymptotic 95% Confidence Interval	
						Lower Bound	Upper Bound
LGALS3	0.832	0.041	<0.0001	90.9%	65.6%	0.752	0.913
LGALS3 LOOCV	0.783	0.047	<0.0001	70.5%	78.7%	0.691	0.875
LGALS3+BIRC5	0.841	0.04	<0.0001	79.5%	78.7%	0.763	0.919
LGALS3+BIRC5 LOOCV	0.809	0.046	<0.0001	77.3%	78.7%	0.720	0.898
Multiplex	0.895	0.031	<0.0001	70.5%	93.4%	0.834	0.955
Multiplex LOOCV	0.823	0.042	<0.0001	65.9%	90.2%	0.74	0.906

Figure 2 The diagnostic performance of the best individual marker, two-marker set and multiplex biomarker model. A and B represents ROC curves of the best individual marker, two-marker set and multiplex model and their LOOCV ROC curves, respectively. C, D, E - Scatter dot plot of LGALS3 RQ, LGALS3 and BIRC5 RQ sum and Multiplex model with the best cut off shows how many benign nodules, malignant nodules and thyroid cancer subtypes are misclassified. In table are the best individual marker, two-marker set and multiplex biomarker model and their LOOCV model ROC curve AUC data and sensitivity and specificity at the best cut-off point.

The future efforts will be focused on adding marker genes to the multiplex model that would enable detecting other molecular subtypes of thyroid cancer, testing the feasibility of the biomarker assay in FNA biopsies and validating the assay in a large multicenter clinical trial.

Conclusions

mRNA expression analysis of 8 candidate marker genes - *BIRC5, CCND1, CDH1, CITED1, DPP4, LGALS3, MET* and *TFF3* in paired nodular and relatively normal thyroid tissue specimens of 105 consecutive patients undergoing thyroid surgery demonstrated that all of them except *CDH1* are differentially expressed between the normal and malignant thyroid tissues and between benign and malignant nodules, and *LGALS3* had the highest diagnostic value for the discrimination of malignant and benign thyroid nodules on a single gene basis. An application of multivariate logistic regression analysis resulted in the generation of a multiplex biomarker model based on *LGALS3, BIRC5, TFF3, CCND1, MET* and *CITED1* that outperformed a single marker or two marker gene-based models by increasing the specificity, which is a prerequisite for the development of clinically applicable biomarker assay. The next step will be to test the feasibility of this assay on FNA biopsies and to validate it in a larger cohort of patients.

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Author details

[1]Faculty of Medicine, University of Latvia, Raina Bulvaris 19, Riga, LV1586, Latvia. [2]Department of Molecular Biology, Latvian Biomedical Research and Study Centre, Ratsupites 1,Riga, LV1067, Latvia. [3]Surgery Clinics, Pauls Stradins Clinical University Hospital, Pilsonu 13, Riga, LV1002, Latvia. [4]Oncology department, Riga Eastern Clinical University Hospital, Hipokrata 2, Riga, LV1038, Latvia.

Authors' contributions

KD participated in the designing of the study, collecting of clinical data and drafting the manuscript. AA carried out the gene expression analysis, performed the statistical analysis and helped to draft the manuscript. JV, ZN and JT performed thyroid surgery and collected the clinical material. DA participated in the gene expression analysis. AL participated in the design of the study and drafted the manuscript. VP designed and coordinated the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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3.2 Functional role of TFF3 in thyroid cancer

Trefoil factor 3 is required for differentiation of thyroid follicular cells and acts as a context-dependent tumor suppressor

A. Ābols[1;2], K. Ducena[2], D. Andrejeva[1], L. Sadovska[1;2], E. Zandberga[1], J. Vilmanis[3], Z. Narbuts[3], J. Tārs[4], J. Eglītis[4], V. Pīrāgs[2;3] and A. Linē[1]

[1] Latvian Biomedical Research and Study center, Rātsupītes street 1, Riga, LV - 1067, Latvia;

[2] University of Latvia, Raiņa boulevard 19, Riga, LV - 1586, Latvia;

[3] Pauls Stradins University Hospital, Pilsoņu street 13, Riga, LV- 1002, Latvia;

[4] Latvian Oncology Center, Riga Eastern Clinical University Hospital, Hipokrāta street 2, Riga, LV - 1038, Latvia

Corresponding author: Artūrs Ābols, Latvian Biomedical Research and Study center, Rātsupītes street 1, Riga, LV - 1067, Latvia. E-mail: arturs@biomed.lu.lv

Running title: The role of TFF3 in the development of thyroid cancer

Key words: thyroid cancer, follicular cells, estrogen signaling

Abstract

Trefoil factor 3 (TFF3) is overexpressed in a variety of solid epithelial cancers, where it has been shown to promote migration, invasion, proliferation, survival and angiogenesis. On the contrary, in the majority of thyroid tumors, it is downregulated, yet its role in the development of thyroid cancer remains unknown. Here we show that TFF3 exhibits strong cytoplasmic staining of normal thyroid follicular cells and colloid and the staining is increased in hyperfunctioning thyroid nodules, while it is decreased in all thyroid cancers of follicular cell origin. By meta-analysis of gene expression datasets, we found that in the thyroid cancer, conversely to the breast cancer, the expression of TFF3 mRNA was downregulated by estrogen signaling and confirmed this by treating thyroid cancer cells with estradiol. Forced expression of TFF3 in anaplastic thyroid cancer cells resulted in decreased cell proliferation, clonal spheroid formation and entry into the S phase. Furthermore, it induced acquisition of epithelial-like cell morphology and expression of the differentiation markers of thyroid follicular cells and transcription factors implicated in the thyroid morphogenesis and function. Taken together, this study provides the first evidence that TFF3 may act as a tumor suppressor or an oncogene depending on the cellular context.

Introduction

Trefoil factor 3 (TFF3) is a member of trefoil factor family consisting of three secreted low-molecular weight peptides that share overlapping functional properties and a structurally conserved trefoil domain composed of 42 - 43 amino acids folded in a compact 3-loop structure [1]. According to the Oncomine data (Roth normal and Shyamsundar normal datasets), TFF3 is highly expressed in thyroid gland, intestines, respiratory tract, salivary gland, myometrium and cervix [2]. It is predominantly secreted by mucous-producing cells and has been shown to interact with mucins to increase the viscosity and elasticity of the mucous covering gastrointestinal tract [3;4]. TFF3 has an essential role in the maintenance and repair of the intestinal mucosa after the damage induced by various cytotoxic agents [5;6] and has been implicated the regulation of epithelial cell migration [7;8], cilliogenesis and differentiation of airway epithelial cells [9]. It has been shown to modulate intestinal permeability by upregulating tight junction associated proteins ZO-1, occluding and claudin-1 in the PI3K/Akt-dependent manner [10]. Moreover, it has also been found to be expressed in neurons and activated microglial cells and to elicit neuroprotective effects in experimental cerebral ischemia/reperfusion injury [11].

A growing body of evidence suggests that TFF3 has a crucial role in the development and progression of cancer. It has been found to be overexpressed at the mRNA and protein level in a variety of solid cancers including breast, gastric, prostate and colon cancer, and endometrial and hepatocellular carcinoma [12-15]. In these cancers TFF3 is oncogenic and has been shown to promote migration, invasion, proliferation, survival and angiogenesis [12;16;17]. Interestingly, in breast cancer, the expression of TFF3 is upregulated by estrogen [18], while in prostate cancer it is regulated by androgen receptor signaling [16]. Upregulation of TFF3 confers metastatic phenotype and correlates with a lower survival rate in gastric, breast and rectal cancer [19-21], whereas it seems to be associated with good prognosis in endometrial adenocarcinoma [15].

On the contrary, TFF3 is downregulated in a subset of thyroid cancers that are derived from follicular epithelial cells. Decreased expression of TFF3 mRNA in thyroid follicular carcinoma was first reported by Takano T et al [22] and this finding was subsequently reproduced by several groups in follicular, papillary and anaplastic carcinomas [23-26] and validated at protein level by an immunohistochemical study [27]. TFF3 mRNA is one of the most promising biomarkers for pre-operative diagnosis of thyroid cancer and the development of a biomarker assay based on the measuring of TFF3 mRNA level in thyroid aspirates is ongoing [28]. However, to the best of our knowledge, the functional role of TFF3 in the development of thyroid cancer has not been elucidated so far.

We hypothesized that TFF3 is required for the differentiation or normal function of thyroid follicular cells and its downregulation may contribute to thyroid tumorigenesis. Therefore, at first we analyzed the expression pattern of TFF3 in normal thyroid tissues and a variety of hyperfunctioning and malignant thyroid nodules by immunohistochemistry, compared its expression level in the thyroid and breast cancers by qRT-PCR and assessed its regulation by estrogen signaling in thyroid cancers. Next, we examined the effects of forced expression of TFF3 in an anaplastic thyroid carcinoma cell line 8305C, where the expression of endogenous TFF3 is downregulated, and breast cancer cell line MCF7, where TFF3 has been shown to be oncogenic [12]. We show here that in the thyroid cancer, on the contrary to the breast cancer, TFF3 expression is downregulated in response to estradiol and the restoration of TFF3

83 expression decrease cell proliferation and spheroid formation capacity, and induces expression of
84 thyroid differentiation markers.

Materials and Methods

Patients and tissue specimens

A cohort of 105 consecutive patients with benign or malignant thyroid nodules undergoing total or partial thyroidectomy at the Latvian Oncology Centre and Pauls Stradins University Hospital during the period 2009-2010 was described previously [26]. This cohort included 61 patient with follicular adenoma (FA), 33 patients with papillary thyroid carcinoma (PTC), 5 - with medullar thyroid carcinoma (MTC), 3 - with anaplastic thyroid carcinoma (ATC) and 3 - with follicular thyroid carcinoma (FTC).

Paired breast cancer and adjacent normal tissue specimens were obtained from 33 breast cancer patients undergoing surgery in the Latvian Oncology Centre. The tissue specimens were macroscopically dissected by a histopathologist during surgery and stored in RNALater (Ambion, Life Technologies, CA, USA) at -20°C till processing. Tissue sections were evaluated by an experienced pathologist, and the diagnosis was established according to standard histopathological criteria. One of the specimens was diagnosed as $ER^+/PR^+/HER2^+$, 5 as $ER^+/PR^-/HER2^-$, 10 as $ER^+/PR^+/HER2^-$, 5 as $ER^+/PR^-/HER2^-$, 7 as $ER^-/PR^-/HER2^+$ and 5 as triple negative breast cancers.

The tissue specimens were collected after the patients' informed consent was obtained in accordance with the regulations of Ethics Committee of the Institute of Experimental and Clinical Medicine, University of Latvia.

Immunohistochemical analysis

Immunohistochemical staining of the Thyroid diseased tissue array (A401, ISU ABXIS, Seoul, Korea) and Thyroid cancer tissue array (A210, ISU ABXIS, Seoul, Korea) was performed according to the manufacturer's protocol. The tissue arrays where deparaffinized and rehydrated in xylene and ethanol followed by quenching of peroxidase. Epitope retrieval was performed by heating the slides in microwave for 15 min in 10mM sodium citrate buffer. Next, the tissue sections were incubated with anti-TFF3 antibody (ab57752, monoclonal, Abcam, Cambridge, UK) at the dilution 1:300 overnight at +4°C and the antibody binding was detected with anti-mouse IgG secondary antibody conjugated with peroxidase (A2554, Sigma - Aldrich, MO, USA) at the dilution 1:50. Colorimetric detection was performed with DAB (Sigma - Aldrich, MO, USA) followed by counterstaining with hematoxylin. Further, the slides where dehydrated, cleared with xylene and coverslips were mounted with Canadian Balsam (Sigma - Aldrich, MO, USA).

Each tissue core was evaluated by assessing three parameters - the intensity of staining, the percentage of stained cells and the pattern of staining (cytoplasmic or follicular lumen). The intensity of immunoreactivity was scored as follows: negative - 0, weak staining - 1, intermediate staining - 2, strong staining - 3. The percent of stained cells was scored as follows: negative - 0, up to 25% - 1; up to 50% - 2, up to 75% - 3 and up to 100% - 4. For statistical analysis, the mean intensity and score of stained cells and ±SD was calculated for each type of thyroid nodules.

Plasmid constructs

Human TFF3 cDNA (GeneBank accession number NM_003226) was amplified by PCR from normal thyroid tissue cDNA using the forward NheI-TFF3 F 5' - AGCTAGCATGAAGCGAGTCCTGAGCTGC – 3 and the reverse EcoRI-TFF3 R 5' - AGAATTCCTAAGAAGGTGCATTCTGCAAAGC - 3' primers. The obtained PCR product

was digested with FastDigest EcoRI and NheI (Fermentas, Thermo Fisher Scientific, MA, USA) and cloned into the mammalian expression vector pIRESneo3 (Clontech Laboratories, CA, USA).

Cell culture and transfection

The human anaplastic thyroid carcinoma cell line 8305C and human mammary carcinoma cell line MCF7 were purchased from European Collection of Cell Cultures (ECACC, UK) and maintained according to the manufacturer's protocols. To generate stable TFF3-expressing 8305C and MCF7 cells, 1×10^5 cells were seeded in 24-well plate and transfected with pIRESneo3-TFF3 or empty pIRESneo3 vector as a control using TurboFect transfection reagent (Fermentas, Thermo Fisher Scientific, MA, USA). After 8 weeks of selection with G418 (Clontech Laboratories, CA, USA), stable transfectants were obtained and designated as 8305C-TFF3, 8305C-pIRES, MCF7-TFF3 and MCF7-pIRES, respectively. MCF7 cells were cultured in DMEM supplemented with GlutaMax (Gibco, Life Technologies, CA, USA), 10% FBS (Gibco, Life Technologies, CA, USA), 1% NEAA (non essential amino acid solution) (Sigma - Aldrich, MO, USA) and 800 μg/ml G418. 8305C cells were cultured in DMEM supplemented with GlutaMax, 10% FBS, 1% NEAA and 500 μg/ml G418.

For the growth curve analysis, the cells were seeded at a density 0.5×10^4 cells per well in 24-well plates and cultured for 10 days. Triplicate wells were trypsinized and the cells were counted using hemocytometer every 24 hours.

For estradiol stimulation experiments, the 8305C cells were seeded at a density 1×10^6 cells per well in 6-well plates in triplicates in serum-free DMEM/F12 medium containing 1X B-27 supplement Minus AO (Life Technologies, Carlsbad, CA, USA), EGF (20ng/ml, R&D Systems, MN, USA), bFGF (10ng/ml, R&D Systems, MN, USA). After 48 hours the cells were treated with 10pM, 1nM, 100nM or 1μM β-estradiol (Sigma Aldrich, St. Louis, MO) or 96% ethanol as a control for 72 hours.

Spheroid forming assay

For spheroid cultures, single-cell suspensions of 8305C-TFF3, 8305C-pIRES, MCF7-TFF3 and MCF7-pIRES cells were plated at a density 2×10^3 cells per ml in 96-well ultra-low attachment plates (Corning, NY, USA) in serum-free DMEM/F12 medium containing 1X B-27 supplement Minus AO, EGF (20ng/ml), bFGF (10ng/ml) and 1% methylcellulose (Sigma-Aldrich, St. Louis, MO, USA). After 7 days in culture, the tumour spheroids containing at least 16 cells were counted under an inverted microscope.

Proliferation assay

Cell proliferation was assessed using Click-iT® EdU assay (C10424; Life Technologies, CA, USA) according to the manufacturer's protocol. Briefly, the cells were seeded at density $5\text{x}10^5$ cells per well in 6-well plate in triplicates, grown until 80-90% confluence and incubated with 30 μM 5-ethynyl-2´-deoxyuridine for one hour. The cells were harvested and, after the fixation and permeabilization, stained with Alexa Fluor 647 azide. The DNA content was analyzed by flow cytometry on the BD FACSAria II cell sorter (BD Biosciences, CA, USA) using BD FACSDiva software (BD Biosciences, CA, USA).

RNA extraction, cDNA synthesis and qRT-PCR

Homogenization of tissue specimens, extraction of RNA, cDNA synthesis and quantification of TFF3 mRNA expression in the tissues was performed as previously reported [26]. The total RNA from cell lines (~2×10^6 cells) was extracted by using mirVana™ PARIS kit (Ambion, Life Technologies, CA, USA) and treated with DNAse according to the manufacturer's instructions. cDNA was synthesized by random hexamer priming from 3µg of total RNA by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, CA, USA). Quantitative RT-PCR was carried out using 2µl of 1:10 diluted cDNA reaction mixtures, ABSolute Blue™ SYBR green Low ROX (Thermo Fisher Scientific, MA, USA) on ABI7500 sequence detection system (Applied Biosystems, Life Technologies, CA, USA). The primer sequences and concentrations used for qRT-PCR are available upon request. To normalize the expression data, a normalization factor was calculated for each cDNA from the expression values of two reference genes - POLR2A and PGK1 by using geNorm Win3.4 software. The reference genes were selected as the most stable among 7 commonly used housekeeping genes (GAPDH, ACTB, POLR2A, TUB3A, TBP, YWHAZ, PGK1). All reactions were carried out in triplicates.

Western Blot analysis

Protein was isolated simultaneously with the total RNA from ~2×10^6 cells by using mirVana™ PARIS kit according to the manufacturer's instructions, precipitated with isopropanol and dissolved in 100 µl 8mM Urea in 60 mM Tris-HCl and 1% SDS. Ten micrograms of total protein were applied per lane and separated by 10% SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes and stained with Ponceau S solution to ensure the normalisation control for protein loading. The membranes were destained, blocked with 5% (w/v) fat-free milk and then incubated with the following primary antibodies: TFF3 (ab57752, monoclonal, Abcam, Cambridge, UK) (1:1000), E-cadherin (sc8426, monoclonal, Santa Cruz Biotechnology, TX, USA) (1:1000) and β-actin (ab8224, monoclonal, Abcam, Seoul, Korea) (1:2000). After washing, the membranes were incubated with peroxidase - conjugated goat anti-mouse secondary antibody (A2554, polyclonal, Sigma - Aldrich, MO, USA) (1:3000), washed and processed with ECL Select Western Blotting Detection Reagents (GE Healthcare, WI, USA) according to manufacturer's instructions.

Statistical analyses

All experiments were repeated at least twice and were performed in triplicates. For continuous variables, the data were analyzed using Student's unpaired two-tailed *t* test. The data are represented as mean ± SD. $P<0.05$ was considered as statistically significant. For the TFF3 mRNA expression analysis in tissues, the data are represented as median with 25^{th} and 75^{th} percentiles and whiskers that represents minimum and maximum values.

For meta-analysis of thyroid cancer gene expression data sets, data were collected from publicly available microarray data sets in the Oncomine database. Gene expression values were log2-transformed, median centered per array, and SD normalized to one per array. Statistical analysis was done by using Student's two- tailed unpaired *t* test.

The statistical analyses were performed with Genex (Multid, Sweden) and Graph- PadPrism 5 (GraphPad, USA).

Results

Expression of TFF3 mRNA in thyroid and breast cancers

In a previous study aiming to identify gene expression biomarkers that could discriminate malignant from benign thyroid nodules, we found that TFF3 mRNA expression was downregulated in 85% (28/33) PTC cases, 67% (2/3) FTC, 100% (3/3) ATC cases and in 66% (40/61) benign nodules when compared to the adjacent normal tissue, while it was upregulated in 80% (4/5) MTC cases [26]. In order to compare the expression level of TFF3 in cancerous and normal thyroid tissues with that in breast cancer where it has been previously found to be overexpressed [12], its mRNA level was measured in 33 paired breast cancer and adjacent relatively normal tissue specimens by qRT-PCR (Fig. 1a). The results showed that in ER^+ breast cancers, the mean TFF3 mRNA level was 3.9-fold upregulated in comparison to the normal breast tissues, while no differences were found in ER^- cancers. Despite to the overexpression, in ER^+ cancers the mean TFF3 mRNA level was 4.5-fold lower than in papillary and follicular thyroid cancers and similar to that in ATC. TFF3 expression level in normal thyroid tissues was 70.6-fold higher than that in normal breast tissues.

Expression of TFF3 mRNA is downregulated by estradiol in thyroid cancer

The expression of TFF3 has been shown to be induced by estradiol in estrogen-responsive cell lines [18]. Therefore, we examined the effect of estradiol on the expression of endogenous TFF3 in the 8305C cells. The cells were treated with 10 pM - 1 μM β-estradiol for 72 hours and the TFF3 mRNA expression was analyzed by qRT-PCR. The estradiol treatment decreased expression of TFF3 in the 8305C cells in a dose-dependent manner (Fig. 1b). To further clarify the relationship between the TFF3 expression and estrogen signaling in thyroid cancers, a meta-analysis of three thyroid cancer microarray data sets – Giordano Thyroid [29], Vasko Thyroid [30] and Bittner Thyroid (unpublished) available in the Oncomine database [2] was performed. Log-transformed intensity values of TFF3, ESR1 and ESR2 were median centered and scaled, and dichotomized according to the expression level of ESR1 or ESR2 and the median intensity values of TFF3 were compared in the groups with high vs low ESR1 and ESR2 expression. The data demonstrated an inverse correlation between TFF3 and ESR1 expression in follicular cell-derived cancers but not in the MTC and benign adenomas in Giordano and Vasko Thyroid data sets (Fig. 1c). No such correlation was found for TFF3 and ESR2 (data not shown).

Immunohistochemical analysis of TFF3 expression in thyroid tissues

The immunohistochemical analysis of TFF3 expression was carried out using a tissue array comprising a total of 72 thyroid tissue specimens representing Graves' disease, granulomatous thyroiditis, Hashimoto thyroiditis, adenomatous hyperplasia, Hürthle cell adenoma, follicular adenoma, various thyroid cancers and normal thyroid tissues. The results are summarized in Table 1 and the representative IHC staining patterns are shown in Fig. 2. In line with the mRNA expression data, all normal tissue samples stained positive for TFF3 with the intensity score of at least 2. TFF3 staining was localized mainly in follicular lumen and cytoplasm of follicular cells (Fig. 2a). Hyperfunctioning nodules such as Graves' disease (Fig. 2b) and toxic multinodular goiter exhibited very strong luminal staining, while in the euthyroid nodules, except for the MTC, the staining was weak or absent (Fig. 2 c-e). In all cases of PTC and FTC, most cells showed weak cytoplasmic staining, both ATC samples were negative for TFF3 expression, whereas MTC demonstrated strong cytoplasmic staining in most of the cells (Fig. 2f).

Forced expression of TFF3 in 8305C cells decreases cell proliferation and entry into S phase

To investigate the functional role of TFF3, human anaplastic thyroid cancer cell line 8305C and mammary carcinoma cell line MCF7 were stably transfected with TFF3 expression vector pIRESneo3-TFF3 or an empty pIRESneo3 vector as a control. The 8305C cells were selected due to low level of endogenous TFF3 expression, whereas MCF7 cells were selected as it has been previously reported that forced expression of TFF3 in these cells increased cell proliferation, survival and enhanced anchorage-independent growth [12]. Increased expression of TFF3 mRNA and protein in 8305C-TFF3 and MCF7-TFF3 cells relatively to the control cells was verified by qRT-PCR and Western blot analysis (Fig. 3 a, b).

The growth curve analysis showed that 8305C-TFF3 cells had significantly decreased proliferation rate and/or increased cell death rate compared with the control cells (Fig. 3 c, d). Concordantly, EdU proliferation assay revealed that forced expression of TFF3 in 8305C cells reduced the number of cells in S- phase from 26.3% to 5.8% (p=0.0053). At the same time, MCF7-TFF3 cells demonstrated increased proliferation rate and S-phase entry (Fig. 3 e) as this has been reported previously [12].

Next, the effect of forced TFF3 expression on invasion of 8305C cells was examined by transwell invasion assay QCM ECMatrix (Millipore, Merck, MA, USA) with 8 μm pore inserts. After 48 hours of incubation using 10% FBS as chemoattractant, no significant differences in the number of cells that have traversed the cell-permeable membrane were observed between the 8305C-TFF3 and the control cells (data not shown).

Forced expression of TFF3 in 8305C cells reduces spheroid-forming ability

To assess the effect of TFF3 on the multicellular spheroid-forming ability, the 8305C-TFF3, MCF7-TFF3 and the respective control cells were plated at low density in methylcellulose-containing serum-free medium in ultra-low attachment plates. In these conditions, cells grew as non-adherent, 3D multicellular spheroids. Clonal origin of the spheroids was confirmed by co-culture of DiD and DiO-labelled cells (data not shown). This assay showed that 8305C-TFF3 cells had significantly decreased number of spheroid-forming cells compared to the control cells (p=0.002), while the spheroid-forming ability of MCF7-TFF3 cells was significantly increased compared to MCF7-pIRES cells (p=0.01) (Fig. 3 f).

Forced expression of TFF3 in 8305C cells induces differentiation

After transfection, we observed changes in the cell morphology in the TFF3 expressing 8305C cells (Fig. 3 g). The 8305C-TFF3 cells had morphological features typical of epithelial cells, while 8305C-pIRES cells retained spindle-like cell morphology. Next, we analyzed the effects of forced TFF3 expression in 8305C cells on the expression of a panel of genes involved in the differentiation and normal function of thyroid tissues and regulation of proliferation by qRT-PCR (Table 2). In agreement with the decreased proliferation rate, the mRNA level of CCNE1 encoding G1/S-specific cyclin E1 was significantly decreased in 8305C-TFF3 cells compared to 8305C-pIRES cells. Concordantly with the epithelial-like morphology, 8305C-TFF3 cells had 9.2-fold higher expression of CDH1 encoding E-cadherin and slightly decreased expression of FN1 encoding fibronectin 1. Upregulation of E - cadherin was also confirmed at the protein level (Fig. 3b). At the same time, the expression of functional thyroid differentiation markers – TPO, TSHR, THRB, SLC26A4 and SLC5A5 were induced while TG was downregulated in the TFF3-expressing cells. Two of the transcription factors implicated in the control of transcription of these genes and thyroid morphogenesis – FOXE1 and WWTR1 were 24 to 44.2- fold

upregulated, while NKX2-1 and PAX8 were expressed at low levels. The expression of ESR1 and ESR2 encoding estrogen receptors was not significantly alerted by TFF3.

Discussion

Although thyroid is the predominant site of TFF3 production and decreased TFF3 mRNA expression is well established biomarker of thyroid cancer, to the best of our knowledge, its role in the normal thyroid function and the development of thyroid malignancies had not been investigated so far. Immunohistochemical analysis of TFF3 in normal thyroid tissues revealed strong staining of follicle colloid that was increased in hyperfunctioning thyroid nodules thus suggesting that TFF3 may have a role in the processing of thyroid hormones, protecting the follicular surface from the damage by H_2O_2 or regulating the viscosity of the colloid. TFF3 mRNA and protein levels were decreased in all thyroid cancers derived from follicular cells, particularly in ATCs that were negative in IHC analysis, thus suggesting an association with decreased hormone production and/or differentiation of follicular cells. At the same time, TFF3 expression was increased in MTC that develops from parafollicular cells, which are of different embryonic origin than follicular cells [31]. The follicular cells originate from the endoderm, while parafollicular cells are believed to originate from the neural crest cells [32]. Hence, distinct expression pattern of TFF3 in these types of thyroid cancers further support a hypothesis that the role of TFF3 in tumourigenesis is strictly dependent on the cell type.

In breast cancer, the transcription of TFF3 has been shown to be stimulated by estradiol [18] and in line with this it was found to be overexpressed in ER^+ breast cancers and implicated in anti-estrogen resistance [12]. Here we confirm this finding and show that in thyroid cancer, on the contrary, TFF3 expression is downregulated by treatment with estradiol. Several lines of evidence indicate that estrogens contribute to the pathogenesis of thyroid cancer. The incidence of thyroid cancer is more than 5-fold higher in women of reproductive age than in men, while the gender ratio declines after the menopause. The serum levels of estradiol were found to be significantly higher in women with differentiated thyroid cancer than in cancer-free controls [33]. Both, normal and malignant thyroid tissues express functional estrogen receptors, however cancers commonly gain ERα expression and lose ERβ expression that has been shown to stimulate their growth and is associated with a more aggressive phenotype [34;35]. Furthermore, a recent study demonstrated that estrogens stimulated growth and simultaneously inhibited the differentiation of thyroid stem/progenitor cells [36]. A meta-analysis of the gene expression data set revealed a strong inverse correlation of TFF3 and ESR1 expression but not ESR2 expression thus supporting the evidence that ESR1 signaling downregulates TFF3 expression in thyroid cancer. In prostate cancers with *ERG* rearrangements resulting in the expression of transcription factor ERG under an androgen-regulated promoter, TFF3 expression has been shown to be regulated by ERG [16]. The effect, however, was opposite in hormone-naïve and castration-resistant cancers – EGR had an inhibitory effect on TFF3 expression in hormone-naïve cancers while TFF3 was upregulated in castration-resistant state. This provides evidence that the expression of TFF3 is regulated by androgen receptor signaling in a cell context-dependent manner. Analysis of the TFF3 promoter region using MatInspector (Genomatix software suite) revealed the presence of two putative estrogen response elements at the positions 566-588 and 803-825 and the binding site for AHR-ARNT heterodimer at the position 886-910 upstream from the transcription start site. ARNT has been recently shown to act as a cell-type specific coactivator of ERα transcription complex in MCF7 cells and as a corepressor in ECC-1 endometrial carcinoma cells [37]. Hence we speculate that the differential regulation of TFF3 by

estrogen signaling in various cells depends on a crosstalk between ERα and cell-type specific transcription coactivators and corepressors such as ARNT.

Next, we show that the restoration of TFF3 expression in anaplastic thyroid cancer cell line with low level of endogenous TFF3, results in the decrease of cell proliferation and entry into the S-phase. Concordantly, a gene expression analysis revealed that *CCNE1* encoding cyclin E1, which is required for cell cycle G1/S transition, was downregulated in TFF3-expressing cells. Furthermore, TFF3 expressing 8305C cells showed a reduced ability to form multicellular spheroids. Formation of clonal spheroids is a characteristic of cancer stem cells that has been shown to correlate with the tumorigenicity *in vivo* in various cancers, therefore this assay has been used both for the isolation of putative cancer stem cells and assessing their self-renewal capacity [38-41]. A recent study of four ATC cell lines demonstrated that spheroid-forming cells expressed the stem cell markers NANOG and OCT4, were highly tumorigenic and metastasized more aggressively in than monolayer-derived cells in an orthotopic mouse model of thyroid carcinoma [42] . Hence, our study suggest that the restoration of TFF3 expression in the 8305C cells may induce differentiation of cancer stem cells and reduce their self-renewal capacity. In accordance with this, we observed that TFF3-expressing cells changed the cell morphology displaying features of epithelial cells and upregulated the expression of E-cadherin, a cell- cell adhesion molecule that is downregulated in the majority of thyroid cancers and has been shown to contribute to invasion and metastasis [43]. Furthermore, the gene expression analysis demonstrated that the forced TFF3 expression in 8305C cells induced the expression of a number of genes that are implicated in the regulation of thyroid gland function and are required for synthesis of the thyroid hormones T_3 and T_4. Thyroperoxidase (TPO) is a thyroid-specific enzyme required for iodination of tyrosine residues of thyroglobulin (TG). *SLC5A5* encodes for the sodium-iodide symporter (NIS) required for uptake of iodide from the blood circulation and *SLC26A4* encodes for pendrin mediating iodide efflux into the follicular lumen. *TSHR* encodes for the receptor for thyroid stimulating hormone – the primary physiological regulator of thyroid function, which is required for normal thyroid differentiation [44;45]. *THRB* encodes for thyroid hormone receptor β, which has been recently shown to act as a tumor suppressor through downregulation of the AKT-mTOR-p70 S6K pathway [46]. In line with this, the dominant negative *THRB* mutant mice have been shown to develop spontaneous follicular thyroid carcinoma [47]. Furthermore, we show there that two of the transcription factors - FOXE1 and WWTR1 that are responsible for the expression of thyroid-specific genes and thyroid morphogenesis were significantly upregulated by TFF3 [48;49]. These data strongly suggest that TFF3 is required for normal function of thyroid follicular cells and the forced expression of TFF3 in undifferentiated cancer cells at least partially restores the differentiated phenotype. This is in agreement with the study of Pierre LeSimple et al [9] showing that besides motogenic and antiapoptotic activity, TFF3 plays a major role in human airway epithelial regeneration by promoting ciliated cell differentiation.

Taken together, this study for the first time demonstrates that in thyroid cancer, on the contrary to the breast cancer, the expression of TFF3 is downregulated by estrogen receptor signaling and TFF3 is implicated the control of follicular cell differentiation and proliferation thus suggesting that its downregulation in thyroid tumors is not merely a consequence of the loss of normal function of follicular cells but contributes to the thyroid tumorigenesis. Hence, this study provides evidence that TFF3 may act as a tumor suppressor or an oncogene depending on the cellular context. We speculate, the dual nature of TFF3 in different cell types may be explained

by formation of different protein complexes, different post-translational modifications or other regulatory mechanisms similarly as it has been demonstrated for orphan nuclear receptor Nur77, transcription factor KLF6 [50;51] and menin [52]. For instance, inactivation of menin is associated with heritable and sporadic endocrine cancers, whereas it also has been shown to be critically required for the formation of macromolecular complex containing proto-oncogene MLL that participates in Hox gene expression and pathogenesis of leukemia [52]. Furthermore, this study suggests that estrogen – TFF3 signaling pathway could represent a novel therapeutic target for the treatment of anaplastic thyroid cancer.

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Figure legends

Fig. 1. TFF3 mRNA expression levels in thyroid and breast cancer tissues and its regulation by β-estradiol. (a) qRT-PCR analysis of TFF3 mRNA expression level in thyroid cancer specimens (33 PTC, 3 FTC, 5 MTC, 3 ATC), 105 adjacent normal thyroid tissues, 61 benign nodule specimens, 12 ER- and 21 ER+ breast cancer specimens with corresponding normal breast tissues. Boxes represent the 25^{th} and 75^{th} percentiles; whiskers represent the minimum and maximum values. Statistical significance was calculated using Mann - Whitney U test. (b) qRT-PCR analysis of endogenous TFF3 mRNA level in 8305C cells treated with 10 pM up to 1μM β-estradiol for 72h. (c) Meta-analysis of TFF3 expression of three thyroid cancer microarray data sets available in Oncomine database. TC1 -dataset of Giordano Thyroid Cancer samples [30], TC2- dataset of Vasko Thyroid Cancer samples [31], TC3 - dataset of Bittner Thyroid Cancer samples (unpublished), TA1 - dataset of Giordano Thyroid Adenoma samples [30]. Log-transformed, median-centered values were dichotomized according to ESR1 expression level. ** p<0,001, *** p<0,0001.

Fig 2. Immunohistochemical analysis of TFF3 expression in benign, inflammatory and malignant thyroid diseases and normal tissues. Representative examples demonstrate mean intensity scores and staining pattern in thyroid nodule types and non - neoplastic thyroid. (a) Normal thyroid specimen, (b) Graves' disease specimen, (c) Papillary thyroid cancer, (d) Follicular thyroid cancer, (e) Anaplastic thyroid cancer, (f) Medullar thyroid cancer. Black lines designate scale bars of 10 μm.

Fig. 3. Forced expression of TFF3 affects the cell morphology and reduces proliferation and spheroid-forming ability. (a) Relative quantity (RQ) of TFF3 mRNA in 8305C-TFF3, 8305C-pIRES, MCF7-TFF3 and MCF7-pIRES cells. (b) Western blot analysis of TFF3 and E-cadherin in the cell lysates. β actin was used as a loading control. (c) Growth curves for MCF7-TFF3 and MCF7-pIRES cells. (d) Growth curves for 8305C-TFF3 and 8305C-pIRES cells. (e) EdU proliferation assay in 8305C-TFF3, 8305C-pIRES, MCF7-TFF3 and MCF7-pIRES. (f) Spheroid formation assay of 8305C-TFF3, 8305C-pIRES, MCF7-TFF3 and MCF7-pIRES cells (g) Representative images of morphological differences between 8305C- TFF3 and 8305C-pIRES cells. Black lines designate scale bars of 100 μm. *** p<0,0001, ** p<0,001, * p<0,01.

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605 **Table 1.** Immunohistochemical analysis of TFF3 expression in thyroid tissues

Nodule type (number of specimens)	Mean intensity ±SD	Main localization pattern	Mean score of stained cells ±SD
Graves' disease (n=5)	3±0.44	Cytoplasm and follicular lumen	4±0.44
Granulomatous thyroiditis (n=4)	2±0.5	Cytoplasm and follicular lumen	3±0.5
Hashimoto thyroiditis (n=6)	2±1.16	Cytoplasm and follicular lumen	2±1
Adenomatous hyperplasia (n=6)	2±1.16	Cytoplasm and follicular lumen	1.5±1
Hürthle cell adenoma (n=6)	2±0.88	Cytoplasm	4±0.83
Follicular adenoma (n=10)	2±0.84	Cytoplasm and follicular lumen	2.5±1.25
Non - neoplastic (n=8)	2±0.53	Cytoplasm and follicular lumen	2.5±1
PTC (n=11)	1±0.64	Cytoplasm	2±1.16
MTC (n=5)	3±0	Cytoplasm	4±0
FTC (n=5)	1±0.7	Cytoplasm	2±1
ATC (n=2)	0±0	Not detected	0±0

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609 **Table 2.** qRT-PCR analysis of gene expression ratio in 8305C-TFF3 and 8305C- pIRES cells

Target Name	Ratio (TFF3/pIRES)	P value
Thyroid hormone production		
TPO	10.70	0.0275
TSHR	3.55	0.0562
SLC5A5	6.72	0.0054
THRB	3.00	0.0064
SLC26A4	2.25	0.0086
TG	0.38	0.0023
Transcription factors		
FOXE1	24.05	0.0016
WWTR1	44.20	0.001
PAX8	0.91	0.0792
NKX2-1	ND	-
Linage markers		
CDH1	9.18	0.0161
FN1	0.87	0.0032
Estrogen receptors		
ESR1	0.93	0.2835
ESR2	0.77	0.0084
Cell cycle		
CCND1	1.13	0.0198
CCNE1	0.49	0.0003
CDK1	0.88	0.4683
CDK2	0.73	0.0134
CDK4	0.80	0.0004
CDKNIB	1.10	0.0789

610 Average of three replicates represents fold change for each gene. A value above 1 indicates
611 increased gene expression and value below 1 indicates decreased gene expression in 8305C-
612 TFF3 cells relative to 8305C-pIRES. ND - expression is under detection limit.

Fig. 1 Download full resolution image

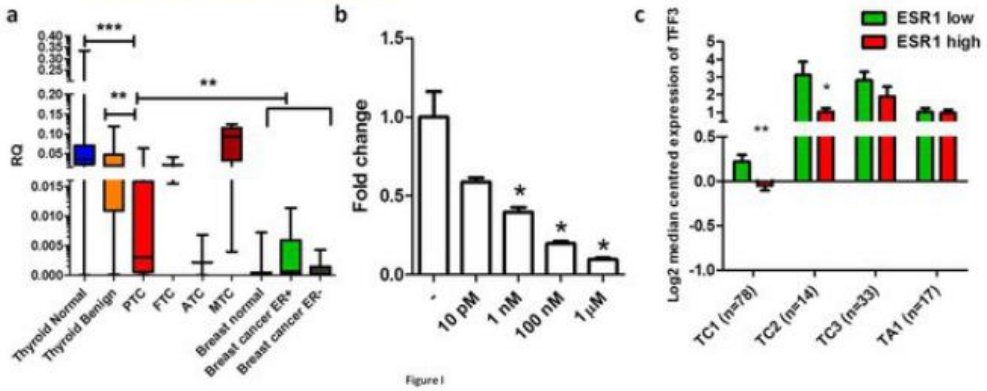

Figure I

Fig. 2 Download full resolution image

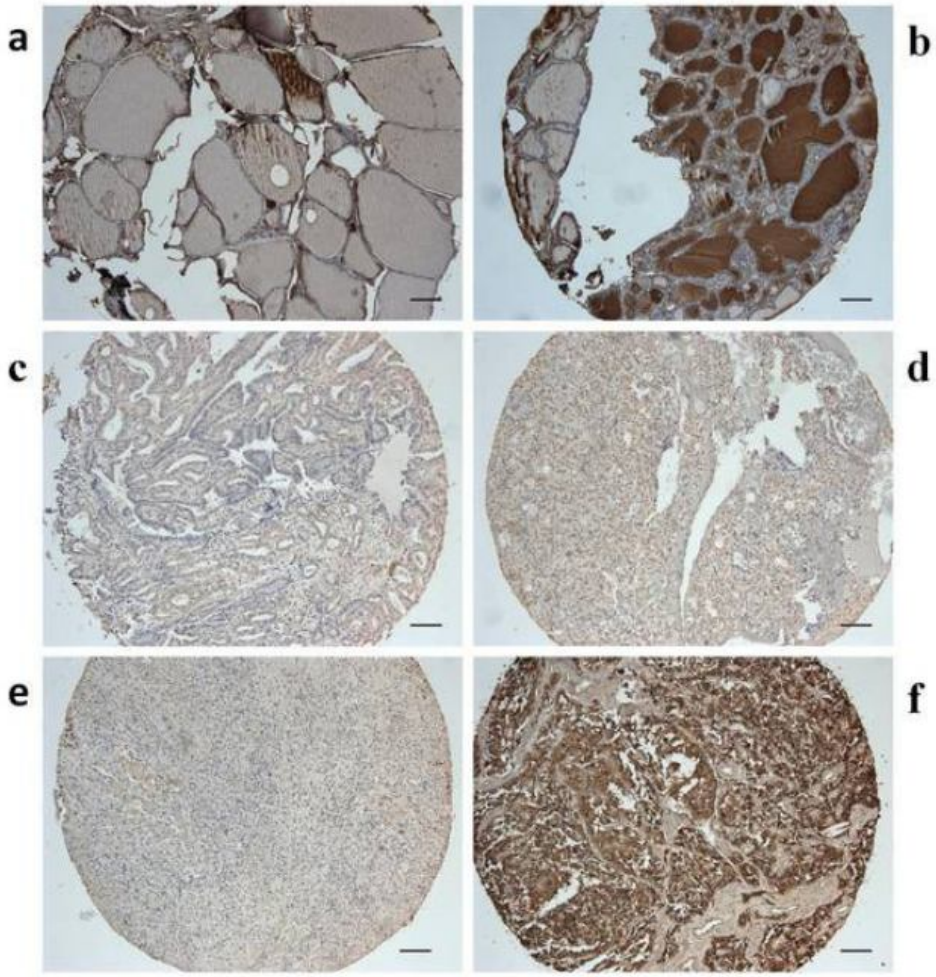

Figure II

Fig. 3 Download full resolution image

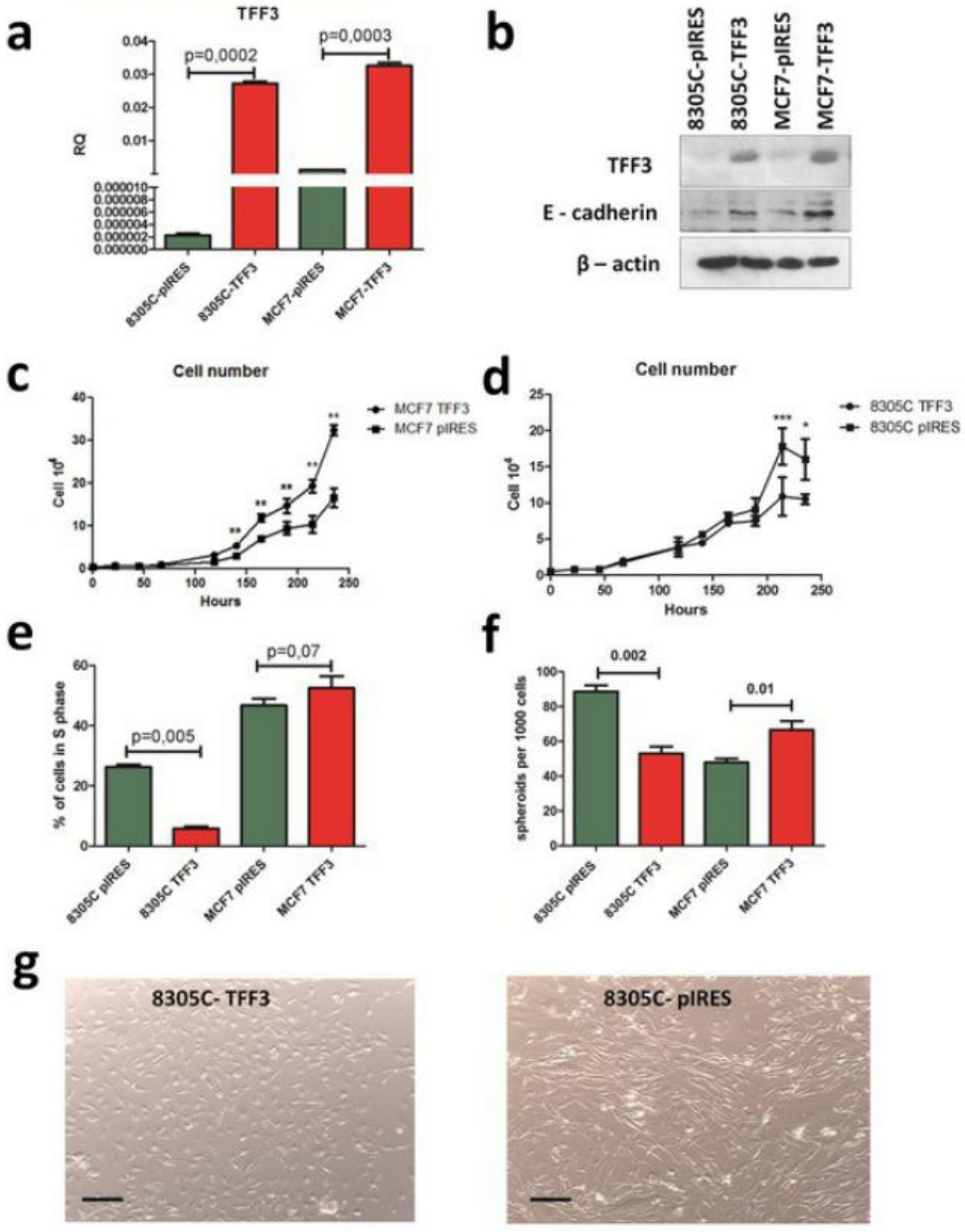

Figure III

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Survey of autoantibody responses against tumor-associated antigens in thyroid cancer

A. Abols[a,b,*], K. Ducena[b], P. Zayakin[a], K. Silina[a], Z. Kalnina[a], L. Sadovska[b], J. Tars[d], J. Vilmanis[c], Z. Narbuts[c], J. Eglitis[d], V. Pirags[b,c] and A. Line[a]
[a]*Latvian Biomedical Research and Study Centre, Riga, Latvia*
[b]*University of Latvia, Riga, Latvia*
[c]*Pauls Stradins University Hospital, Riga, Latvia*
[d]*Latvian Oncology Center, Riga Eastern Clinical University Hospital, Riga, Latvia*

Abstract.
BACKGROUND: Autoantibodies against tumor-associated antigens (TAAs) have been shown to serve as highly specific serological biomarkers for the diagnosis of various solid cancers. Although the autoimmunity against thyroid tissue specific antigens has been studied extensively, so far, the autoantibody responses against common TAAs such as cancer-testis antigens (CTAs), mutated or differentiation antigens have not been comprehensively analyzed in patients with thyroid cancer.
OBJECTIVE: The current study aims to characterize the frequency of autoantibody responses against common TAAs in patients with thyroid cancer and benign thyroid nodules.
METHODS: A phage-displayed antigen microarray comprising 65 TAAs was produced and tested with sera from 53 patients with thyroid cancer, 90 patients with benign thyroid nodules and 96 cancer-free individuals, 100 melanoma, 54 breast cancer and 14 lung cancer patients as controls.
RESULTS: A panel of 6 TAAs was identified that preferentially reacted with sera from patients with thyroid cancer. The top ranked antigen in this panel was GAGE1 eliciting autoantibody response in 6% of patients with thyroid cancer but not with benign nodules, whereas no reactivity to other CTAs was detected in the sera from patients with thyroid cancer.
CONCLUSIONS: Although six TAAs, including one CTA, showed thyroid cancer-associated reactivity, overall, spontaneous humoral immune responses against TAAs are rare in thyroid cancer and their utility for the development of non-invasive assay for the differential diagnosis of thyroid nodules is limited.

Keywords: Phage-displayed antigen microarray, autoantibodies, thyroid nodules, thyroid cancer, cancer-testis antigens, biomarker

Abbreviations

ATC	anaplastic thyroid carcinoma;
AUC	area under the curve;
CTA	cancer-testis antigen;
FA	follicular adenoma;
FNAB	fine needle aspiration biopsies;
FTC	follicular thyroid carcinomas;
HD	healthy donors;
MTC	medullar thyroid carcinoma;
PTC	papillary thyroid carcinoma;
ROC	receiver operating characteristic;
SEREX	serological analysis of recombinant cDNA expression libraries;
TAA	tumor-associated antigen.

*Corresponding author: Arturs Abols, Latvian Biomedical Research and Study Centre, Ratsupites Str 1, LV-1067, Riga, Latvia. Tel.: +371 67808208; Fax: +371 67442407; E-mail: arturs@biomed.lu.lv.

1. Introduction

The prevalence of palpable thyroid nodules ranges from 3 to 7% in the general population. The great ma-

jority of them are benign lesions such as hyperplastic follicular adenomas, multinodular goiter, cysts and thyroiditis, however 5 to 8% of nodules prove to be malignant [10]. The most common type of malignant lesions is papillary thyroid carcinoma (PTC) accounting for ∼80% of thyroid cancers, followed by follicular thyroid cancer (FTC). Both PTC and FTC are derived from thyroid follicular cells and differ mainly in the mode of metastatic spread (lymphatic and haematogenous spread, respectively) yet both are relatively indolent tumors with 5-year survival rates > 90%. In contrary, undifferentiated or anaplastic thyroid carcinoma (ATC) accounting for up to 2% of thyroid cancers is a highly aggressive and lethal cancer with 5-year survival rate below 1–17% [19]. A minority of thyroid carcinomas are derived from parafollicular cells (or C-cells) and are referred to as medullary carcinomas with 5-year survival rate of ∼80% [19].

Currently, the diagnostic assessment of thyroid nodules is based on the cytological examination of US-guided fine needle aspiration (FNA) biopsies, however the results may be non-informative in ∼15–30% of cases due to an inadequate sampling and the lack of highly specific, measurable cytological criteria [7, 19,21]. Most patients with indeterminate cytological findings are subjected to diagnostic surgery, however only ∼30% of these nodules prove to be malignant in the post-operative histological evaluation [2]. Recently, several gene-expression classifiers that allow highly accurate discrimination between benign and malignant nodules have been identified [2,8,9], however they also rely on sampling of the nodule by FNA. Hence, considering the high prevalence of nodular thyroid disease, the development of a blood test for the early detection of thyroid cancer and the differential diagnosis of nodules would be of utmost clinical relevance.

Autoantibodies against tumor-associated antigens (TAAs), due to their specificity, stability in the sera and robust detection methods, represent attractive targets for the development of serological tests for the detection of cancer. Cancer-specific autoantibody signatures with diagnostic relevance have been identified in a variety of solid cancers, including prostate, lung, breast, ovarian and gastric cancer [4,6,26–28]. Several of these studies demonstrated that cancer-testis antigens (CTAs) – antigens that are normally expressed only in germ line cells and aberrantly expressed in a wide range of cancer types, are among the most frequent autoantibody targets and therefore are of the highest diagnostic value [14]. Furthermore, these antigens represent important immunotherapeutic targets and the detection of spontaneous autoantibody responses could be useful for the stratification of patients for antigen-specific immunotherapy, particularly in the cases when the tumor tissue is not available for the expression analysis. However, in thyroid cancer, the humoral immune response against CTAs and other common TAAs has not been comprehensively analyzed so far. In the current study, we produced a 75-feature phage-displayed antigen microarray containing 19 CTAs and other common tumor antigens such TP53, SOX2, TERT etc. and exploited it for the autoantibody profiling in patients with thyroid cancer, benign thyroid nodules and cancer-free controls and compared the profiles with those in patients with melanoma and breast and lung cancer.

2. Materials and methods

2.1. Study population

Pre-treatment blood samples from 143 consecutive patients undergoing total or partial thyroidectomy at the Latvian Oncology Centre and Pauls Stradins University Hospital were collected during the period 2009–2011. The diagnosis was established according to standard histopathological criteria. Ninety of the patients were diagnosed with follicular adenoma (FA), 38 with papillary thyroid carcinoma (PTC), 6 with medullar thyroid carcinoma (MTC), 3 with anaplastic thyroid carcinoma (ATC) and 6 with follicular thyroid carcinoma (FTC). Serum samples from 96 age and gender matched cancer-free healthy individuals and 54 patients with breast cancer, 100 patients with melanoma and 14 patients with non-small cell lung cancer (NSCLC) were provided by the Genome Database of Latvian Population. The blood samples were collected in venous blood collection tubes with clot activator and gel for serum separation (Becton Dickinson, NJ, USA) and stored at +4°C until processing. The samples were centrifuged and aliquoted within 48 hours and stored at −80°C. Clinico-pathological characteristics of the study population are provided in Table 1. Study procedures were approved by the Central Committee of Medical Ethics of Latvia and the ethical committee of the University of Latvia, Institute of Experimental and Clinical Medicine. The blood samples were collected after the patients' informed consent was obtained.

Table 1
Clinical and pathological characteristics of the study population

Characteristics	Thyroid cancer $n = 53$	Benign nodule $n = 90$	Healthy controls $n = 96$	Melanoma $n = 100$	Breast cancer $n = 54$	NSCLC $n = 14$
Gender						
Male	9	13	50	51	–	13
Female	44	77	46	49	54	1
Age at diagnosis						
Median (range)	60 (24–83)	55 (24–80)	63 (34–83)	60 (22–91)	57 (40–72)	62 (44–77)
Histological type or staging						
	Subtype:			Stage:	Subtype:	Stage:
	PTC, $n = 38$	FA, $n = 90$	N/A	I, $n = 20$	ER+ $n = 26$	III $n = 14$
	FTC, $n = 6$			II, $n = 21$	ER- $n = 17$	
	MTC, $n = 6$			III, $n = 22$	N/A $n = 11$	
	ATC, $n = 3$			IV, $n = 37$		

N/A, not available.

2.2. Production and processing of phage displayed antigen microarrays

For the production of 75-feature TAA microarray, a panel of 65 recombinant T7 phage clones previously selected from phage-displayed cDNA expression libraries and 10 non-recombinant phage clones was assembled and simultaneously amplified to high titre ($\sim$2–10 $\times$ 10^8 pfu/μl) in *E. coli* BLT 5616 cells using 96 well culture plates (Whatman, UK). The microarrays were produced and processed as described before [27]. Briefly, the lysates were arrayed in duplicates onto nitrocellulose-coated 16-pad FAST slides (Maine Manufacturing, ME, USA) using a QArray Mini microarrayer (Genetix, UK). The slides were dried, blocked with 7% (w/vol) milk powder in TBS, 0.05% Tween 20, incubated with 80 μl of 1:200 diluted patients' sera that were preabsorbed with UV-inactivated *E.coli*- phage lysates for 2 h, washed 4 times in TBS, 0.5% Tween 20 for 15 min, and then incubated with monoclonal anti-T7 tail fiber antibody (Novagen, SanDiego, CA, USA). Next, the microarrays were incubated with Cy5 labelled goat anti-human IgG antibody (1:1500) and Cy3 labelled goat anti-mouse IgG antibody (1:3000) (Jackson ImmunoResearch, PA, USA) for 45 min, then washed in TBS, 0.5% Tween 20, rinsed with distilled water and dried by centrifugation. A reference serum was included in each series of experiments. The arrays were scanned at 10 μm resolution in PowerScanner (Tecan, Switzerland) with 532 and 635 nm lasers, the results were recorded as TIFF files and the data were extracted using GenePix software. The obtained data were further analyzed using an *ad hoc* program composed in R language.

2.3. Microarray data processing and statistical analysis

The microarray data were processed and analyzed as described before [27]. Briefly, the mean Cy5 and Cy3 signals were background subtracted, averaged between replicates, and the Cy5/Cy3 ratios were calculated for each antigen. In order to eliminate variations introduced by the custom production of microarrays and variable background intensities of different sera, the data were median-centred and scaled across the slides. A cutoff value for defining sero-postive antigens in each field was defined as the mean signal intensities of all negative control spots (non-recombinant T7 phage) plus 3 standard deviations (SD). Fisher's exact test was applied to determine the level of significance for the binominal frequency data.

Then the rank (R) for each antigen was calculated, taking into account the signal intensity (I) and frequency of reactivity (N) with sera from cancer patients (Ca) compared to healthy control sera, using the following formula:

$$R_i = \left(\sum \frac{I_{Cai}}{N_{Cai}}\right) - 2\left(\sum \frac{I_{HDi}}{N_{HDi}}\right)$$

Coefficient 2 was introduced in this formula in order to decrease the rank of antigens reacting with sera from healthy controls. Only the antigens that received positive ranking were analyzed further. Finally, a score (S) for each serum was calculated by summing up the intensities of positively ranked antigens as follows: $S = \sum_{i=1}^{n} \sqrt{R_i} \times I_i$. The non-parametric Mann-Whitney U test was used to compare the serum scores between two independent groups of samples. The receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) was calculated to evaluate the diagnostic performance of the serum scores.

3. Results

3.1. Antigen selection and generation of TAA microarray

In a previous study, we applied T7 phage display-based SEREX technique to identify a comprehensive set of antigens that elicit autoantibody responses in patients with gastric, breast and prostate cancer and melanoma and this resulted in the identification of over 1500 distinct antigens, including well-known tumor antigens, such as CTAs, previously uncharacterized antigens and novel artificial peptides [17]. The recombinant phage particles were used for the production of antigen microarrays that were tested with sera from 240 patients with various cancers and 100 cancer-free controls to obtain the serum-reactivity pattern for each antigen. In the current study, a panel of 65 TAAs reacting with at least 2% of sera from patients with gastric or prostate cancer or melanoma but not with healthy controls was assembled. The panel included 19 different phage clones representing various regions or splice variants of 12 X-chromosome CT (CT-X) antigens (CTAG1B, CTAG2, GAGE1, MAGEA1, MAGEA3, MAGEC1, MAGEC2, DDX53, CSAG2, PAGE2, SPANXA2 and SSX2) and 10 clones representing 7 non-X CT antigens (HORMAD1, BAGE, SPAG8, SPAG17, ARMC3, DDX43, THEG), and 14 clones representing mutated antigens such as TP53, overexpressed or differentiation antigens such as TYR, SOX2, ERBB2, HER2, BIRC5, ANXA11, GOLGA6L2, TERT and LRRC50. The remaining 22 phage clones encoded artificial peptides that are likely to represent mimotopes. The nature of the antigens they represent is not known as they may mimic protein as well as non-protein antigens of cancer or normal cells or various pathogens. Ten non-recombinant phage clones were included in the panel as negative controls.

3.2. Autoantibody profiling

To define autoantibodies with a potential diagnostic significance, the 75-feature TAA microarray was tested with sera from 53 patients with thyroid cancer, 90 patients with benign thyroid nodules and 96 cancer and autoimmune disease-free healthy individuals, 100 melanoma, 54 breast and 14 lung cancer patients as controls. Examples of reactivity patterns for sera from patients with thyroid cancer and melanoma are shown in Fig. 1A. After excluding low-quality spots, centering and scaling of the data, a cutoff discriminating between sero-positive and negative antigens was calculated in each array. The cutoff has been previously experimentally validated by plaque assay for 3 antigens – CTAG1B, HORMAD1 and SPAG17 [27]. Next, the antigens were ranked according to the signal intensity above the cutoff and higher frequency of reactivity with sera from cancer patients (all types of cancer) than cancer-free controls as described in "Materials and Methods" section. In total, 23 antigens reacted preferentially with the sera from cancer patients in this cohort and received positive ranking, while 12 antigens reacted at similar frequency and/or intensity with the sera from cancer patients and healthy controls and the remaining antigens didn't react with any of these sera and were excluded from further analysis. The frequency of IgG reactivity against positively ranked antigens is shown in Table 2. The percentage of samples in each group reacting with at least one of these antigens is shown in Fig. 1B. The top ranked antigens were CTAG2 (LAGE-1) and CTAG1B (NY-ESO-1) that elicited the autoantibody responses in up to 27% of patients with stage IV melanoma and 7% of patients with NSCLC, followed by SPAG8, two artificial peptides and the N-terminal epitope of TP53. However, only 6 of these antigens reacted with sera from thyroid cancer patients. This panel included GAGE1, COPS4 and 4 artificial peptides (Fig. 1C). Next, the "serum score" was calculated for each serum sample by summing up the signal intensities above the cutoff for all 23 antigens that had received positive rating as described in "Materials and Methods". The serum score in patients with thyroid cancer ranged from 0 to 4.27 (mean 0.79), while it was 0 to 3.12 in healthy controls (mean 0.095), ($p = 5.2 \times 10^{-5}$) and 0 to 5.64 (mean 0.34) in patients with benign follicular adenoma ($p = 0.02$) (Fig. 1D). To evaluate the diagnostic performance of the serum score, ROC curve analysis was performed and the cutoffs on the ROC curves were determined using Youden's index approach (Table 3). This demonstrated that the serum score could discriminate between thyroid cancer and healthy controls with AUC of 0.61 (95% CI = 0.55–0.66) and had sensitivity of 25% and specificity of 97%. However, 21% of patients with benign FA had positive serum scores and the specificity dropped to 90%, when patients with thyroid cancer were compared to those with FA thus showing that this biomarker set has a limited utility for the differential diagnosis of thyroid nodules.

Table 2
Frequency of autoantibody responses against TAAs with cancer-associated reactivity, %

Clone #	Name	NCBI RefSeq (aa position)	PTC $n = 38$	FTC $n = 6$	ATC $n = 3$	MTC $n = 6$	ThyCa $n = 53$	FA $n = 90$	HD $n = 96$	NSCLC $n = 14$	BC $n = 54$	Melanoma I $n = 20$	Melanoma II $n = 21$	Melanoma III $n = 22$	Melanoma IV $n = 37$
342	CTAG2	NM_172377.4 (1–104)	0	0	0	0	0	0	0	7	0	0	0	9*	27*
268	CTAG1B	NM_001327.2 (1–104)	0	0	0	0	0	0	0	0	0	0	0	9*	27*
28	SPAG8	XM_005251438.1 **1–92**	0	0	0	0	0	1	0	7	4	0	0	0	5
1382	**Artificial peptide**	**28 aa**	**3**	**0**	**0**	**0**	**2**	**1**	**0**	**0**	**0**	**0**	**5**	**5**	**5**
5	**Artificial peptide**	**21 aa**	**5**	**0**	**0**	**0**	**4**	**2**	**0**	**7**	**2**	**0**	**0**	**0**	**0**
1725	TP53	NM_001276696.1 **(1–20)**	0	0	0	0	0	0	0	7	6*	0	0	0	0
326	**GAGE1**	**NM_001040663.2 (1–90)**	**5**	**0**	**0**	**17**	**6***	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**3**
1464	ARMC3	NM_001282747.1 (244–290)	0	0	0	0	0	1	0	0	0	0	0	0	8*
60	Hybrid HNRPH1-RPS2	XM_005265902.2 (182–237)/ NM_002952.3 (265–293)	0	0	0	0	0	8*	1	14*	6	0	5	9	0
1799	SPANX-A2	NM_145662.2 (1–41)	0	0	0	0	0	1	0	0	0	0	0	0	8*
87	**COPS4**	**NM_001258006.1 (15–136)**	**3**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**5**	**0**	**0**
1428	DDX53	NM_182699.3 (31–210)	0	0	0	0	0	0	0	0	0	5	0	0	3
276	SSX2	NM_001278702.1 (1–113)	0	0	0	0	0	0	0	0	0	0	0	0	5
275	SSX2	NM_001164417.2 (1–185)	0	0	0	0	0	0	0	0	0	0	0	0	5
352	MAGEC1	NM_016249.3 (195–368)	0	0	0	0	0	0	0	0	0	0	0	0	5
1419	CSAG3	NM_001129826.1 **(10–108)**	0	0	0	0	0	0	0	0	4	0	0	0	0
357	CTAG1B	NM_001327.2 (84–180)	0	0	0	0	0	0	0	0	0	0	0	0	5
264	MAGEA1	NM_004988.4 (1–129)	0	0	0	0	0	0	0	0	0	0	0	0	3
1454	MAGEA1	NM_004988.4 (1–65)	0	0	0	0	0	0	0	0	0	0	0	0	3
1262	**Artificial peptide**	**33 aa**	**3**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
1418	PAGE2B	NM_001015038.2 (1–69)	0	0	0	0	0	0	0	0	0	0	0	5	0
1730	TP53	NM_001276696.1 **(82–96)**	0	0	0	0	0	0	1	0	9*	5	0	0	0
1237	**Artificial peptide**	**24 aa**	**11**	**0**	**33**	**0**	**9**	**8**	**3**	**0**	**0**	**0**	**0**	**0**	**0**

ThyCa, thyroid cancer (all types); BC, breast cancer; HD, healthy controls; *Statistically significant difference in comparison to healthy control sera (Fishers' exact test $p < 0.05$).

Table 3
Diagnostic performance of the serum score

Characteristics	ThyCa vs HD	ThyCa vs FA	Melanoma vs HD	NSCLC vs HD	BC vs HD
Number of antigens	6	6	20	7	6
Mean serum score	0.79	0.34*	2.70	2.57	1.14
AUC	0.61	0.57	0.68	0.66	0.63
AUC significance	5.2e-05	0.02	2.7e-09	0.00031	2.7e-06
Sensitivity, %	25	25	35	36	28
Specificity, %	97	90	99	99	98

*Mean serum score in FA.

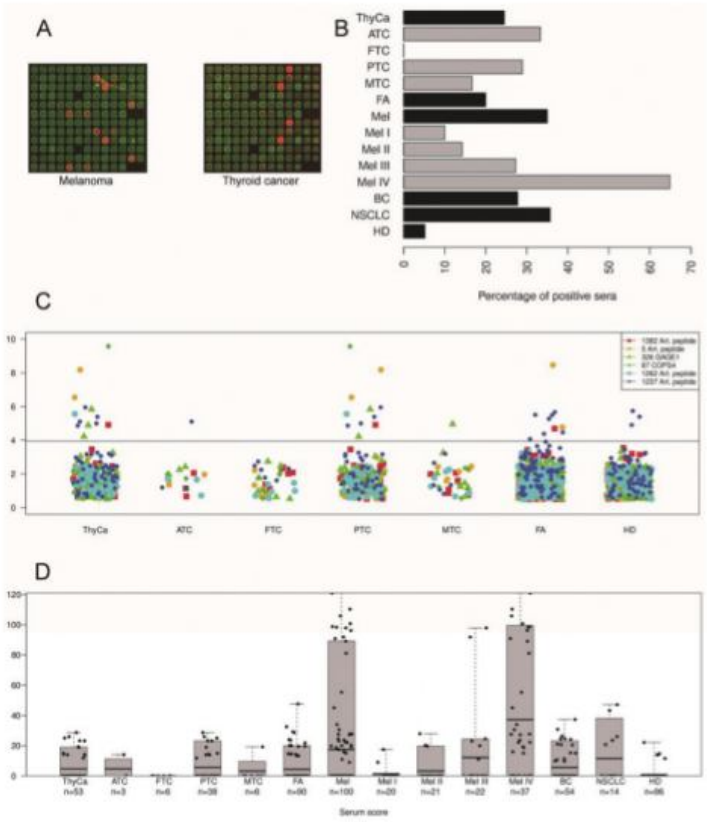

Fig. 1. Analysis of autoantibodies against 65 TAAs in sera from patients with thyroid cancer, benign thyroid nodules, melanoma, breast and lung cancer and autoimmune disease-free healthy individuals. (A) Examples of TAA microarray images showing distinct reactivity pattern in serum from a patient with thyroid cancer and melanoma. (B) The percentage of samples reacting with at least one of the 23 antigens with cancer-associated reactivity in each group of patients and controls. (C) Dot plot showing reactivity pattern of 6 antigens with thyroid cancer associated reactivity. (D) Box plot showing the serum scores based on 23-autoantibody signature in cancer patients and controls. Boxes represent 10^{th} to 90^{th} percentiles, whiskers represent most extreme data points and dots represent individual samples. ThyCa, all thyroid cancer samples; ATC, anaplastic thyroid carcinoma, FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; MTC, medullar thyroid carcinoma; FA, follicular adenoma; Mel, all melanoma samples, Mel I–IV, stage I to IV melanomas; BC, breast cancer; NSCLC, non-small cell lung cancer; HD, cancer and autoimmune disease-free controls.

4. Discussion

CTAs are a heterogeneous group of proteins that are preferentially expressed in immuno-privileged sites of the body such as testis and are aberrantly expressed in a variety of cancers thereby becoming exposed to the immune system [25]. Currently, approximately 200 genes with Cancer-Testis expression pattern have been identified [3]. A comprehensive expression analysis of 153 CT genes revealed that 39 genes are strictly testis-restricted, 14 were expressed in testis and brain, while the rest of them had less restricted expression pattern in somatic tissues and were classified as testis-selective [15]. CT genes are expressed in various types of cancer and, so far, cancer-type restricted CT genes have not been identified. Many CT genes encode antigens that are immunogenic and elicit spontaneous and vaccine-induced immune responses in cancer patients. Others and we have demonstrated that the autoantibody response against these antigens could serve as a very specific biomarker of cancer [11,23,24,27]. For example, autoantibodies against CTAG1B (NY-ESO-1) – one of the most immunogenic CTA, have been found in patients with ovarian, lung, breast, gastric, prostate, esophageal and colorectal carcinomas and lymphomas at frequencies ranging between 0.9 to 25% while rarely detected in cancer-free controls [3]. In a previous study, we found anti-CTAG1B autoantibodies in 17% of patients with gastric cancer, 0.5% of healthy donors but not in patients with gastric inflammatory diseases. Anti-DDX53 autoantibodies were found in 7% of gastric cancer and 2% of peptic ulcer cases but not in healthy donors. A recent study by Shan et al. (2013) showed that the most frequently reactive antigens in NSCLC were CTAG1B, XAGE-1, MAGEC1 and ADAM29 and a combined analysis of responses against these four antigens had a sensitivity of 33% and specificity of 96% [23]. *Early*CDT®-Lung, an autoantibody-based diagnostic assay for lung cancer that has undergone a clinical validation and currently is available for the routine clinical use is based on the measurement of autoantibodies against 7 TAAs (TP53, CTAG1B, CAGE, GBU4-5, SOX2, MAGE A4 and HuD) and has a specificity of 93% and sensitivity of 41% [5,13]. In medullary thyroid cancer, CTAG1B has been shown to be the most frequently expressed CTA eliciting autoantibody responses in 35.7% of patients [22]. However, to the best of our knowledge, the humoral immune response against CTAs in follicular cell-derived thyroid cancers has not been comprehensively studied so far.

In the current study, we assessed the IgG responses against a panel of 19 CTAs in a cohort of 143 consecutive patients with malignant or benign thyroid nodules. We found anti-GAGE1 autoantibodies in 5% of PTC and 17% of MTC cases but not in the patients with benign nodules and cancer-free controls, hence suggesting it may serve as highly specific biomarker for thyroid cancer and may have a clinical utility if combined with other autoantibody biomarkers. However, we did not find autoantibodies against any other CTAs known to elicit humoral response in other types of cancer. At the same time, we detected anti-CTAG1B autoantibodies in 27% of stage IV melanoma patients and 7% of NSCLC patients, anti-SPANX-A2 autoantibodies in 8% of stage IV melanoma cases, anti-TP53 autoantibodies in 7 and 9% of NSCLC and breast cancer patients, respectively, thus confirming that the assay can detected the presence of IgGs with adequate sensitivity and specificity. Considering the fact that thyroid cancer is associated with the presence of concomitant or preexisting autoimmunity against thyroid tissue specific antigens such as peroxidase or thyroglobulin [12], the finding that anti-CTA autoantibodies are rare in the patients with thyroid cancer seems to be surprising. One of the possible explanations could be the relatively slow growth rate and aggressiveness of thyroid cancers. At least in melanoma, there is a striking correlation between the advanced stage and the production of cancer-associated autoantibodies suggesting that the humoral immune response to cancer is associated with the tumor burden and/or the metastatic spread. Hence, it could be possible that the relatively indolent tumors such as PTC and FTC do not trigger B cell activation. Concerning the expression pattern of CTAs in thyroid cancers, gene expression data that are publically available at the Oncomine database [1] suggest that SSX1 - 4 and CTAGE5 are the most frequently overexpressed CTAs in thyroid cancers, however SSX2 and CTAGE5 are also increased in FA. Furthermore, several studies have analyzed the expression of MAGEA family antigens in thyroid cancers showing that MAGEA1 to 6 are significantly overexpressed in papillary thyroid microcarcinoma suggesting that it may be related to the early stage of PTC [20].

Concerning non-CT antigens, we detected anti-COPS4 autoantibodies in 3% of PTC cases. COPS4 is a subunit of COP9 signalosome, a highly conserved protein complex that functions as a regulator in multiple signaling pathways. Previously, we had detected autoantibodies against COPS4 in melanoma patients yet no other evidence on its immunogenicity has been

reported and the mechanisms of its immunogenicity are not clear. Furthermore, we detected autoantibodies against 4 artificial peptides that most likely are mimotopes of other antigens of protein or non-proteinnature. However, the serum reactivity against these antigens was also found in patients with benign thyroid nodules thus limiting their relevance for the differential diagnosis of thyroid nodules.

Recently, two SEREX studies of thyroid cancer have been reported. By immunoscreening of thyroid cancer cDNA expression libraries, Kiyamova et al. [18] identified 15 antigens [18]. Three of these antigens reacted specifically with sera from patients with thyroid cancer, however the frequency of responses ranged from 4 to 8% thus also showing that cancer-specific autoantibodies in thyroid cancer are relatively rare. On the contrary, Izawa et al. [16] identified two ubiquitously expressed antigens – WDR1 and fibronectin 1 and demonstrated that anti-WDR1 autoantibody alone could discriminate between thyroid cancers vs benign nodules and healthy controls with sensitivity of 96.7% and specificity of 91.9% [16]. However, this study was based on a small sample set and the diagnostic value of anti-WDR1 autoantibody must be validated in a larger, independent cohort. Furthermore, the mechanism of its immunogenicity remains to be determined.

Taken together, profiling of autoantibody responses against 65 TAAs in patients with malignant and benign thyroid nodules demonstrated that spontaneous humoral immune response against common TAAs is relatively rare in thyroid cancer in comparison with other solid cancers such as melanoma, breast and lung cancer. This study identified 6 TAAs with a diagnostic relevance, however, in order to develop a clinically applicable biomarker assay more antigens with thyroid cancer-associated reactivity must be identified.

Acknowledgements

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4. Discussion

Historically, before the cytological evaluation of ultrasound guided FNAB was established as a standard diagnostic approach, a diagnostic surgery was applied to thyroid nodules due of their 5 - 15% risk of malignancy. FNAB decreased diagnostic thyroidectomy by half because most of the biopsies are diagnosed as benign in cytological evaluation and according to ATA and AACE guidelines a close clinical and sonographic follow up of cytological benign nodules for six to eighteen months after FNAB is recommended. However, even up to 30% of cases of FNAB are indeterminate due to an inadequate sampling and the lack of highly specific cytological criteria (Cooper et al., 2009). Usually, patients with indeterminate cytology undergo thyroidectomy and up to 70% prove to be benign in histopathological evaluation. The Bethesda system claims that subdividing these indeterminate cytological evaluations in higher (FN/SFN and SMC) and lower (AUS/FLUS) risk could improve clinical care (Cibas and Ali, 2009). However it appears that this subdivision created less reproducible categorizations and has not solved the problem, thus this differential approach has been questioned (Walts et al., 2012). After thyroid surgery a thirty day mortality can be up to 0.5%, on average 0.2%. Serious or permanent non-lethal complications of thyroidectomy includes hypothyroidism, hypocalcemia, laryngeal nerve damage, re - bleeding and wound infection. Complications are related with total thyroidectomy combined with lymph node dissection and surgeon expertise (Hundahl et al., 2000) and rates form 5 - 10% in USA even up to 37% in Brasil (Sosa et al., 1998;Ernandes-Neto et al., 2012). Furthermore, the treatment costs are increased due the complications. Therefore, the identification of molecular biomarkers of thyroid cancer could improve not only the accuracy of cytological evaluation of FNAB but also reduce the treatment costs and improve the patients' life quality.

One of the most studied biomarker class in thyroid cancer is somatic mutations and gene rearrangements. Yuri E. Nikiforov and group in the end of 2011 published a research where they prospectively analyzed *BRAF* V600E, *NRAS* codon 61, *HRAS* codon 61, and *KRAS* codons 12/13 point mutations and *RET/PTC1*, *RET/PTC3*, and *PAX8/PPARγ* rearrangements in 967 consecutive thyroid indeterminate FNA samples, the biggest cohort of samples published so far, where mutation analyses were done (AUS/FLUS - 653 samples, FN/SFN - 247 samples, and SMC - 67 samples). This mutation and gene rearrangement model reached the highest correlation of mutational analysis in FNA samples and histopathological outcome in specific groups of indeterminate cytology to our knowledge (AUS/FLUS: Sn - 63%, Sp - 99%, PPV - 88%, NPV - 94%; FN/SFN: Sn - 57%, Sp - 97%; PPV - 87%; NPV - 86%; SMC: Sn - 68%, Sp - 96%, PPV - 95%; NPV - 72%). However, this model have to be tested in multicenter study to confirm its diagnostic value before introduced in routine clinical practice. This study also confirms that a panel of somatic mutations and gene rearrangements outperforms single gene mutation or gene rearrangement diagnostic value (Nikiforov et al., 2011).

A number of immunohistochemical marker approaches has been applied to indeterminate cytology specimens in an effort to reclassify these samples including HBME1, FN1, Galectin - 3, CK19 and others. IHC markers are well suited for the detection of classical PTC, however the sensitivity is significantly lower for the detection of follicular variant of PTC and FTC. Furthermore some markers are positive in FA and normal thyroid tissues (Kim and Alexander,

2012). Imortantly, there is a lack of multicenter, prospective, double blinded clinical trials investigating immunohistochemical markers. Also, there is tremendous variability in specimen processing and interobserver interpretation of the results precluding the introduction of protein markers in clinical routine practice. Thus, most pathologists view imunohistochemistry as having a limited role in thyroid diagnosis (Bongiovanni et al., 2012).

A promising biomarker type is gene expression signatures, including non - coding RNA genes. It is clear now that no individual gene has sufficient accuracy to be exploited as an independent diagnostic biomarker, because of cancer inter and intra heterogeneity. However, there is evidence that combined gene expression signatures from a few up to several hundred genes are capable to discriminate malignant tissues from normal tissues, divide cancers into molecular subtypes, predict treatment outcomes and recurrence. Probably, the most widely used gene expression tests are Oncotype Dx Breast Cancer assay - a test for prediction of breast cancer recurrence based on 21 gene expression signature, Oncotype Dx Colon cancer assay – a test for recurrence risk based on 12 gene expression signature and Oncotype Dx prostate cancer assay - a test for cancer aggressiveness based on 17 gene expression signature (Clark-Langone et al., 2007;Albain et al., 2010;Knezevic et al., 2013). There are several approaches to identify gene expression signatures: DNA microarrays, next generation sequencing and RT-qPCR assays. DNA microarrays offer the advantage of simultaneously assessing the relative expression level of thousands of genes with a relatively small amount of total RNA, however DNA microarray measurements are often too variable between different studies and limited in specificity and dynamic range, leading to false positive and false negative biomarker discovery (Knezevic et al., 2013). Next generation sequencing technology offers the whole transcriptome evaluation and *de novo* biomarker discovery, but still it is very expensive method and requires special infrastructure and trained personal in bioinformatics. On the contrary RT-qPCR technology is relatively cheap and offers high accuracy, reproducibility and wide dynamic range, however there is limitation of simultaneously assessing the relative gene expression and it cannot be used for *de novo* biomarker discovery. The RT-qPCR approach was exploited in the development of Oncotype DX tests that was based on testing of 192 candidate gene panel for the breast cancers test and 761 candidate gene panel for the colon cancer test (Clark-Langone et al., 2007;Albain et al., 2010).

Therefore in the original paper I we choose to analyse 8 candidate genes that have been shown to be deregulated and involved in thyroid cancer development (*CDH1, BIRC5, CCND1, CITED1, MET, DPP4; LGALS3, TFF3*) by RT-qPCR approach. This resulted in the generation of a complex model based on 6 gene expression signature (*LGALS3, BIRC5, CCND1, CITED1; MET; TFF3*) that outperformed the single marker and two gene biomarker models (AUC=0.895; Sn - 70.5%; Sp - 93.4% with p value <0,0001) and clearly warrants further studies in FNAB (Ducena et al., 2011). However, *TFF3* is downregulated in thyroid cancer in comparison to benign nodules and normal thyroid tissues and the development of assay that is based on the measurement of downregulated gene expression level in FNAB consisting of various proportions of cancerous and stromal cells is technical challenging. Takano with colleges in 2009 reported that expression level of TFF3 in FNAB after mesh filtration correlated well with that in corresponding tumor tissue. This is probably because thyroid follicular cancer cells tend to form

clusters in FNAB rather than be a single cells, thus infiltrated lymphocytes and blood cells but no tumor cells pass through the mesh (Takano and Yamada, 2009). The development of pre - operative diagnostic test of thyroid cancer based on the measurement of TFF3 expression level in aspirate flush out is now ongoing by applying this mesh filtration method (Yamada et al., 2012). Recently, Alexander EK with colleagues reported a large, prospective multicenter validation study of commercially available gene expression classifier (GEC) developed by *Veracyte*. In this multicenter, double blinded study, the classifier, which is based on the measurement of the expression levels of 167 genes, was applied to classify 265 indeterminate FNA biopsies. The authors demonstrate that thyroid nodules with indeterminate cytopathology and benign result according to *Veracyte* GEC test result have less than 6% likelihood of being malignant (NPV greater than 94%) (Alexander et al., 2012). Another clinical study showed that among 51 endocrinologists at 21 practice sites, when their patient had an indeterminate cytopathology result and a benign GEC result, physicians recommended surgery in only 7.6% of these cases. This represents a 90% reduction in surgeries when compared to the historical average for patients with indeterminate cytopathology result alone (Duick et al., 2012). *Veracyte* GEC, to the best of our knowledge, currently is the only commercial available option for endocrinologists that could help to decide whether to operate a patient with indeterminate cytopathology or not. However, in future the diagnostic value of these biomarker tests could be improved by combining genetic, epigenetic and gene expression signatures and so far there is no such reports on thyroid cancer.

The original paper II is devoted to the exploration of the functional role of TFF3 in thyroid cancer. Although several groups, including ours (Ducena et al., 2011), have observed that the expression level of TFF3 mRNA is downregulated in thyroid cancer and it currently seems to be one of the most promising biomarkers for pre-operative diagnosis of thyroid cancer, to the best of our knowledge, the functional role of TFF3 in the development of thyroid cancer has not been elucidated so far.

Based on previous reports showing that TFF3 is upregulated in several types of cancer while it is downregulated in follicular cell-derived thyroid cancer we hypothesized, that TFF3 is required for the differentiation of follicular cells and normal thyroid functions and it can act as a cell-context dependent oncogene or tumor suppressor gene. In order to test our hypothesis we performed a comparative TFF3 mRNA expression analysis in breast and thyroid cancer tissues and investigated its regulation in response to β - estradiol treatment in thyroid cancer cell line, studied the protein expression analysis in TMA of various normal and diseased thyroid tissues and examined the effects of forced TFF3 expression in thyroid and breast cancer cell lines. Immunohistochemical analysis of TFF3 in normal thyroid tissues revealed a strong cytoplasmic staining of follicular cells and colloid, and the staining was increased in hyperfunctioning thyroid nodules showing that TFF3 may have role in the processing of thyroid hormones.

Next, we showed that TFF3 is overexpressed in ER+ breast cancers compared to ER- cancers and normal breast tissues, confirming previously published results that in breast cancer transcription of TFF3 is stimulated by estradiol. At the same time, meta-analysis of the Oncomine data demonstrated that TFF3 expression was inversely correlated with ESR1 expression in follicular cell-derived thyroid cancer but not in benign nodules and MTC. Furthermore, the treatment of thyroid cancer cells with β - estradiol decreased transcription of

TFF3 in a dose dependent manner suggesting that TFF3 regulation by estrogen signaling depends on the cell-type specific co-activators or co-repressors.

Forced expression of TFF3 in the anaplastic thyroid cancer cells decreased cell proliferation and entry in S phase by inhibiting cyclin E1, while in the breast cancer cells, TFF3 induced proliferation and entry in the S phase. Spheroid forming assay showed that increased TFF3 expression decreases self renewal in thyroid cancer cell lines while it has opposite effect on breast cancer cells. We also demonstrated that forced TFF3 expression in the thyroid cancer cells induced the expression of genes involved in normal thyrocyte functions and transcription factors involved in thyrocyte differentiation and phenotype maintenance. These results suggests that TFF3 may have a role in normal thyroid functions such as processing of thyroid hormones and maintaining differentiation. Considering the fact that forced TFF3 expression in dedifferentiated thyroid cancer cell line partly restored thyrocyte phenotype we hypothesize that TFF3 loss may contribute to thyroid tumorigenesis. Based on these data and previously published results in other cancer types, we propose that TFF3 can act as tumor - suppressor or oncogene in cell context dependent manner. In future studies this pathway could be targeted in order to increase differentiation level of anaplastic cancer, that has no efficient treatment for now and make it more sensitive to radioactive iodine. For example there is studies that shows that genetic mutations or alterations in gene expression in epithelial and hematopoietic cancers may be manifested as maturation arrest of a cell lineage at a specific stage of differentiation. Understanding the signals that control normal development or maintained differentiation may eventually lead us to insights in treating cancer by inducing its differentiation so called differentiation therapy (Sell, 2004).

Another example of well known oncogene, that can act as tumor suppressor in the cellular context dependent manner is Notch. Notch signalling is a highly evolutionarily conserved pathway implicated in diverse functions during embryogenesis and in self-renewing tissues of the adult organism acting as a cell fate determinant (Leong and Karsan, 2006). When Notch receptors interact with ligands, two consecutive proteolytic cleavages of the receptor are initiated, releasing the intracellular portion of Notch to enter the nucleus and activate the transcription of target genes such as *HES* family of transcription factors, *NRARP (*Notch-related ankryin repeat protein), *c-MYC* and *DTX1 (Weng et al., 2006).* Activation of Notch pathway by translocations or mutations has been implicated in various solid tumors, including breast cancer, medulloblastoma, colorectal cancer, non–small cell lung carcinoma (NSCLC) and melanoma (Ranganathan et al., 2011). However, there is growing evidence that increased Notch pathway activity may have growth-suppressive functions in other cells, like hematopoietic cells, skin, pancreatic epithelium and hepatocytes. Viatour with colleagues in 2011 proposed a new tumor suppressor role for Notch pathway in hepatocellular carcinoma (HCC). They produced a mouse model for disease by deleting retinoblastoma (RB) and its two related family members p103 and p170 in mouse liver. This showed that Notch pathway is upregulated in the liver cells suggesting its oncogenic role in HCC development. Nevertheless, the inhibition of Notch signaling in these mice using DAPT (potent γ-secretase inhibitor) led to accelerated HCC development and forced activation of Notch signalling using ICN1 (truncated form of Notch receptor 1) led to cell cycle arrest and apoptosis in primary HCC cells isolated from mice, as well as in human HCC cell

lines, suggesting the potential tumor suppressor role for Notch pathway in HCC (Viatour et al., 2011). Similar results have been published by other authors in B cell malignancies and neuroblastoma (Zweidler-McKay et al., 2005;Zage et al., 2012). In last decade there is an increasing number of reports on proteins that act as tumor suppressor genes or oncogenes depending on the context such as transcription factor KLF4 (Rowland et al., 2005), Runx genes (Cameron and Neil, 2004) and others, suggesting, that binary classification of genes in oncogenes and tumor suppressor genes is incomplete.

While the discovery of new tissue biomarkers would improve the accuracy of NFAB it would not solve the problems associated with the sampling of biopsy such as variable cell counts and proportions. In addition, biopsy is an invasive procedure and cause a discomfort for the patient. Hence, the developments of a reliable serological diagnostic assay that can detected the presence of malignant thyroid nodules would be of very high clinical relevance. Recent efforts to discover blood-based biomarkers of thyroid cancer has resulted in the identification of several cancer-associated molecules, including circulating TSHR, miRNA, autoantibodies against tumor associated antigens, cell-free DNA fragments, mRNAs and various non-coding RNAs. However, since measurements of TSH, no new blood tests for differentiated thyroid cancer have been introduced into routine clinical practice. The main limitations of serum TSH for the evaluation of malignancy risk are substantial differences in the level of TSH among various racial and ethnic groups as well as changes during the aging process.

In 2004, Chinnappa et. al. reported on the use of circulating TSHR mRNA in human blood which is derived from circulating thyroid cancer cells. The authors report that by combining the examination by ultrasound, cytological evaluation of FNAB and measurement of TSHR mRNA, the diagnostic performance reaches sensitivity of 97%; specificity of 88 %; PPV of 88% and NPV of 95% for predicting thyroid cancer. However, this study was carried out in a single institution and an expanded clinical experience from other institutions is needed to verify this approach (Milas et al., 2010;Chinnappa et al., 2004).

In last decade, many studies have provided evidence that miRNAs can be released from different type of cells including cancer cells into the extracellular space. They circulate into all biofluids (blood, salvia, milk, ect) and can be taken up by recipient cells, where they reagulates different pathways resulting in diverse physiological and pathological responses. These results suggests that circulating miRNA may also be used as minimal-invasive biomarkers for cancers including thyroid cancer detection. However, there are only few of these studies was focused on the thyroid cancer serological markers on miRNA level. Yu and colleges in 2012 discovered that three miRNAs (let-7e, miR-151-5p, and miR-222) were significantly increased in PTC patient serum comparing to benign cases and healthy controls. They showed that serum let-7e, miR-151-5p, and miR-222 levels correlates with certain clinicopathological variables, such as nodal status, tumor size, multifocal lesion status and metastasis and it is significantly decreased in PTC patients decreased significantly after tumor excision (Yu et al., 2012a). In line with this, Lee with colleagues showed that miRNA-222 and miRNA 146b levels in tumor tissues were significantly higher in patients with recurrent PTC compared to those without recurrence ($p=0.014$ and $p=0.038$, respectively). These miRNA were also elevated in PTC patients' plasma before surgery compared with healthy donor plasma ($p<0.01$ for both) and were significantly reduced in PTC

patient plasma after thyroidectomy, compared with the plasma collected before surgery ($p<0.03$ for both), suggesting that miRNA-146b and miRNA-222 are associated with PTC recurrence and their level in blood circulation corresponds to presence of PTC (Lee et al., 2013). Although the development of qRT-PCR based multiplex assays for the detection of miRNA in cancer patient blood for diagnostic purposes seems to be relatively simple and straightforward, currently it is still hampered by several technical issues. In contrary to the analysis of gene expression in tissue specimens, currently there is no consensus on housekeeping genes in serum/plasma that could be exploited as internal controls for the normalisation and the obtained results seem to be inconsistent. For example, non-coding RNAs such as U6 snRNA, snoRNAs and rRNAs that are used for tissue miRNA normalisation have been shown to be highly variable in the plasma and readily degradable in serum and therefore unsuitable as internal controls (Chen et al., 2008). Similarly, one of the most frequently used internal control miRNAs – miRNA-16 has been reported as the most strongly deregulated miRNA in the sera from lung cancer patients compared with the healthy controls (Keller et al., 2011). Also, an overrepresentation of RNA species from white or red blood cells in the profile may be an indication that the cells were lysed at some point prior to the RNA isolation. This may be due to incomplete removal of white blood cells and platelets from the sample, and/or due to haemolysis. Even the diameter of syringe needle can affect the miRNA profile in blood sample due to cells contaminating the sample. The presence of cellular RNA species may disturb the serum/plasma microRNA measuring experiment resulting in a distorted and non-reproducible profile. Another issue is that the physiological processes such as pregnancy and a variety of common diseases such as acute or chronic inflammatory diseases, cardiovascular disorders and diabetes have also been associated with the release of miRNAs into the bloodstream (Reid et al., 2011), however the cancer-free control groups in the majority of studies are typically matched by age, gender and smoking status, yet virtually nothing is known about their history of disorders (Blondal et al., 2013).

Another attractive targets for the development of serological tests for the detection of cancer are autoantibodies against tumor-associated antigens (TAAs). Cancer specific autoantibody signatures with diagnostic relevance have been identified in a variety of solid cancers. Several studies demonstrated that cancer-testis antigens (CTAs) – antigens that are normally expressed only in germ line cells and aberrantly expressed in a wide range of cancer types, are among the most frequent autoantibody targets and therefore are of the highest diagnostic value. Furthermore, these antigens represent important immunotherapeutic targets and are useful for the stratification of patients for antigen specific immunotherapy (Heo et al., 2012). However, in thyroid cancer, the humoral immune response against CTAs and other common TAAs had not been comprehensively analyzed so far.

In the original paper III, we explored the repertoire of cancer-associated autoantibodies in patients with thyroid cancer to identify autoantibody based biomarkers for the development of non-invasive biomarker assays. In order to do that we produced a 75-feature phage-displayed TAA microarray and exploited it for the autoantibody profiling in patients with thyroid cancer, benign thyroid nodules and cancer-free controls and compared the profiles with those in patients with melanoma and breast and lung cancer. The microarray comprised 19 different phage clones encoding CTAs and 14 clones encoding mutated antigens and overexpressed or differentiation

antigens. We found anti-GAGE1 autoantibodies in 5% of PTC and 17% of MTC cases but not in the patients with benign nodules and cancer-free controls and anti-COPS4 autoantibodies in 3% of PTC cases thus suggesting these autoantibodies may serve as highly specific biomarkers for thyroid cancer and may have a clinical utility if combined with other autoantibody biomarkers. However, we did not find autoantibodies against any other CTAs. Although we detected autoantibodies against 4 artificial peptides that most likely are mimotopes of other antigens of protein or non-protein-nature, the serum reactivity against these antigens was also found in patients with benign thyroid nodules thus limiting their relevance for the differential diagnosis of thyroid nodules. Taken together, profiling of autoantibody responses against 65 TAAs in patients with malignant and benign thyroid nodules demonstrated that spontaneous humoral immune response against common TAAs is relatively rare in thyroid cancer in comparison with other solid cancers. Thus in order to develop a clinically applicable autoantibody biomarker assay more antigens with thyroid cancer-associated reactivity must be identified.

Taken together, in this doctoral thesis, a multiplex biomarker model based on the measurement of expression levels of 6 genes in thyroid tissues was identified and warrants further studies in FNAB to improve the diagnostics in indeterminate FNAB cases. Next, for the first time we investigated the functional role of TFF3 - one of the most promising thyroid cancer tissue biomarkers known so far, and demonstrated that, in contrary to breast cancer, TFF3 acts as a tumor suppressor gene and is downregulated by estrogen receptor signaling in thyroid cancer thus contributing to tumorigenesis in thyroid cancer. This suggests that this novel TFF3 - estrogen signaling pathway could represent a novel therapeutic target for the treatment of anaplastic thyroid cancer that has very poor prognosis and have no effective treatment for now. Furthermore, in this work for the first time we systematically studied the repertoire of cancer-associated autoantibodies in patients with thyroid cancer, showing that spontaneous humoral immune response against well known tumor antigens, including CTAs, in thyroid cancer is relatively rare comparing to other cancers. Hence, this study provided a deeper insight into the molecular alterations leading to the development of thyroid cancer and revealed biomarkers for the diagnosis and putative therapeutic targets for the development of drugs against thyroid cancer.

5. Conclusions

- mRNA expression levels of 7 out of 8 candidate genes analyzed were significantly different between benign and malignant thyroid nodules (*LGALS3* - $p=10^{-8}$; *TFF3* - p=0,0003; *DPP4* - p=0,0004; *MET*=0,0005; *CITED1*=0,001; *CCND1* - p=0,001, *BIRC5* - p=0,001) and therefore have a diagnostic significance.

- The multiplex biomarker model based on 6 genes (*LGALS3, BIRC5, TFF3, CCND1, MET* and *CITED1*) had a better diagnostic performance for discriminating benign and malignant thyroid nodules (AUC = 0.895, P < 0.0001, 70.5% sensitivity and 93.4% specificity) than a single marker or two marker-based models.

- Expression level of TFF3 is downregulated in the follicular cell-derived thyroid cancers, while it is upregulated in hyperfunctioning thyroid nodules. A putative mechanism of its downregulation in thyroid cancer is estrogen receptor signalling.

- Restoration of TFF3 expression in anaplastic thyroid cancer cells resulted in the decreased cell proliferation, clonal spheroid formation and entry into the S phase, and acquisition of epithelial-like cell morphology and expression of the differentiation markers of thyroid follicular cells and transcription factors implicated in the thyroid morphogenesis and function.

- Six tumor-associated antigens, including GAGE1, that elicit cancer-associated autoantibody responses in patients with thyroid cancer were identified.

- On the contrary to other solid cancers, spontaneous humoral immune responses against Cancer-Testis antigens are rare in thyroid cancer.

Main thesis of defence

Thesis I

The multiplex biomarker model based on measuring the expression level of 6 genes (*LGALS3, BIRC5, TFF3, CCND1, MET* and *CITED1*) is relevant for improving preoperative risk assessment in patients with thyroid nodules.

Thesis II

TFF3 is required for the normal function of thyroid follicular cells and its downregulation in follicular cell-derived cancers is not merely a consequence of the loss of normal function of follicular cells but contributes to the thyroid tumorigenesis. TFF3 can act as a tumor suppressor or oncogene in the cellular context dependent manner.

Thesis III

Spontaneous humoral immune response against common TAAs is relatively rare in thyroid cancer in comparison with other solid cancers and they have a limited utility for the development of non-invasive assay for the differential diagnosis of thyroid nodules.

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