New Molecular Approaches in the Diagnosis and Prognosis of Thyroid Cancer Patients

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Abstract: Thyroid nodules are very common in the adult population, but only a minority of them harbor a malignant lesion. Therefore, the first aim in their clinical evaluation is to exclude malignancy. To date, the fine-needle aspiration cytology (FNAC) represents the main diagnostic tool for the evaluation of thyroid nodules and cervical lymph nodes (CLN) suspected of metastatic disease. It has to be mentioned, however, that FNAC on thyroid nodules suffers from a major diagnostic limit represented by cellular atypias of indeterminate significance, which require the histological diagnosis. Also the FNAC performed on CLN may be a challenging diagnostic category as CLN could harbor metastases from a multiplicity of extrathyroidal malignancies or be affected by several non-tumoral diseases. In addition, inadequate cellularity obtained from both thyroid nodules or CLN prevents diagnosis in about 20% of specimens.

Total thyroidectomy followed by adjuvant therapy with ^{131}I is the treatment of choice for most patients affected by DTC. Although the prognosis of DTC patients is favorable, about 20% of them face the morbidity of disease recurrence and tumor-related deaths. Thus far, the prognosis of these patients still relies on clinic-pathological variables such as patient's age, tumor size, histology, lymph node or distant metastasis, which are not accurate in predicting the long-term outcome. As a consequence, the identification of new molecular biomarkers strictly related to the risk of DTC relapse is highly needed.

In the present review we'll attempt to summarize the recent characterization of new molecular markers able to ameliorate the diagnosis and prognosis of thyroid cancer patients.

Keywords: BRAF, Estrogen receptor, Calcitonin, Cervical lymph node, Gene expression, miRNA, TNM, Thyroglobulin, Urokinase plasminogen activating system.

THYROID CANCER OVERVIEW

Tumors derived from the follicular thyroid cell are the most frequent endocrine malignancy representing in the United States the fifth most common cancer in women [1, 2]. Its annual incidence, about 2.5%, increased over the last two decades, mainly because of the improved ability to diagnose malignant transformation in small non-palpable thyroid nodules [3, 4]. The large majority of epithelial thyroid cancers is represented by the differentiated papillary (PTC) and follicular (FTC) thyroid carcinomas which, following dedifferentiation, are thought to give rise to the highly aggressive and incurable anaplastic thyroid carcinomas (ATC) [5, 6]. Although derived from the same cell type, the different thyroid tumors show specific histological features, biological behavior and degree of differentiation as a consequence of different genetic alterations [7, 8]. In particular, early genetic mutations in thyroid cancer comprise gene rearrangements of tyrosine kinase receptors, such as the RET/PTC and NTRK1 (neurotrophic receptor-tyrosine kinase 1), or activating point mutations of proteins mediating cellular responses to growth and differentiation signals, including RAS and BRAF, or the oncogenic fusion protein PAX8-PPARγ, that suppresses wild-type PPARγ function in a dominant-negative manner [7, 8]. Importantly, the conversion of early-stage thyroid tumors to more aggressive and invasive malignancies occurs through an epithelial-to-mesenchymal transition (EMT), which implies the loss of cell–cell contacts, remodeling of cytoskeleton, and the acquisition of a migratory phenotype [9, 10]. In fact, genetic alterations of integrins, Notch, MET, TGFβ, NF-κB, PI3K, TWIST1 and p21-activated kinase (Pak), related to the EMT, have been identified in PTC progression [10-12].

Thyroid nodules are very common, affecting 19% to 67% of the adult population, but only about 5% of them harbor a malignant lesion [13, 14]. Therefore, the first aim in the clinical evaluation of a thyroid nodule is to exclude malignancy [15]. To date, fine-needle aspiration cytology (FNAC) represents the main diagnostic tool for the evaluation of both palpable and non-palpable thyroid nodules. It has to be mentioned, however, that FNAC suffers from a major diagnostic limit represented by follicular lesions (Thy3), in which the encountered cellular atypias are of indeterminate significance [15, 16]. In this context, we'll below

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describe new molecular approaches claimed to ameliorate the diagnosis of these patients. Also the accurate diagnosis of cervical lymph node metastasis, which is of primary importance for the initial surgical approach as well as for prognostic stratification and follow-up of thyroid cancer patients, relies on FNAC [17-20]. The latter, however, crucially depends on the experience and ability of the cytopathologist, and may be a challenging diagnostic category as CLN could harbor metastasis from a multiplicity of extrathyroidal malignancies or be affected by several non-tumoral diseases [21, 22]. In addition, inadequate cellularity or non-representative sampling, often associated with cystic lymph nodes, prevents diagnosis in about 20% of specimens [23, 24]. Below we'll discuss as the molecular diagnosis performed in the CLN washout may increase the diagnostic accuracy of FNAC.

Total thyroidectomy followed by adjuvant therapy with ^{131}I is the treatment of choice for most patients affected by differentiated thyroid carcinoma (DTC) [1, 13-16]. After that, patient's follow-up includes radioiodine scanning 6-12 months after surgery, periodic ultrasound of the thyroid bed and cervical lymph node compartments, measurement of basal and recombinant human TSH-stimulated thyroglobulin serum level [1, 13-15]. Although the prognosis of patients with DTC is favorable, with 10-years-survival rate of nearly 90%, about 20% of patients face the morbidity of disease recurrence and deaths [1, 2, 15, 25]. The latter, in particular, are observed in patients with poorly differentiated thyroid cancer (PDTC) or ATC [7, 25, 26]. As a consequence, new therapeutic approaches for the most aggressive thyroid cancers are still urgently needed [27-31]. Despite the increasing knowledge of the molecular processes responsible for thyroid cell malignant transformation and cancer progression, to date, the prognosis of thyroid cancer patients still relies on high-risk clinic-pathological variables such as patient's age, tumor size, histology, lymph nodal or distant metastasis [32, 33]. As a consequence, the identification of molecular biomarkers strictly related to the risk of PTC relapse represents an attractive gain [34, 35]. Below we'll summarize recent findings describing the potential prognostic value of different new molecular markers able to refine the prognostic stratification of thyroid cancer patients [34, 35].

NEW MOLECULAR APPROACHES IN THE DIAGNOSIS OF THYROID NODULES

FNAC represents the gold standard in the diagnosis of thyroid nodules because of its diagnostic accuracy, reproducibility and cost effectiveness (15, 16). In particular, FNAC based diagnosis of thyroid nodules is characterized by a sensitivity of 65-98%, specificity of 72-100% and accuracy of 84-95% [36-38]. The Bethesda System for reporting thyroid cytopathology classifies the FNAC outcome in 6 diagnostic categories including: 1) non-diagnostic; 2) benign; 3) atypia/follicular lesion of undetermined significance; 4) follicular neoplasm or suspicious for follicular neoplasm; 5) suspicious for malignancy; 6) malignant [39, 40]. While FNAC diagnosis is very reliable for PTC and ATC, the diagnostic categories 3 to 5 represent a grey zone in which the cytology cannot discriminate malignant (i.e. follicular carcinoma and follicular variant of papillary carcinoma) from benign (i.e. follicular adenoma and nodular adenomatous goiter) tumors [38]. Consequently, in presence of nodules with indeterminate cytology, thyroidectomy is usually required because only the histological evaluation of capsular and vascular invasion can differentiate these neoplasms. Based on histological outcome the category atypia/follicular lesion shows a malignancy risk of 5-15%, that of follicular neoplasm or suspicious for follicular neoplasm of 15-35%, and that of suspicious for malignancy of 60-75%. As a consequence, a consistent number of patients is actually facing needless thyroid surgery. Thus, the identification of parameters related to malignancy is strongly required to avoid morbidity and costs associated to unnecessary surgery. Several alternative diagnostic approaches were put forward in order to overcome the diagnostic boundaries of FNAC, including patient's clinical characteristics, ultrasonography (US) parameters, radionuclide scanning with technetium-99m, positron-emission tomography and identification of single genetic alterations in fine-needle aspiration material. To date, however, none of them turned out to improve significantly the pre-surgical selection of nodules with indeterminate cytology [38-53].

Over the last few years, two different new molecular approaches were proposed to evaluate FNA samples [54, 55]. The first one, introduced by Nikiforov and colleagues, suggested to differentiate benign from malignant nodules based on the detection in FNA samples of the seven most common proto-oncogene mutations encountered in PTC and FTC, namely RET/PTC1, RET/PTC3, $BRAF^{V600E}$, HRAS (codon 61), KRAS (codon 12 and13), NRAS (codon 61) and PAX8/PPARγ [56-59]. It has to be noted in fact that, with the exclusion of RAS, mutation tests for each of these genes possess a very high predictive value for

malignancy. In the larger prospective study, including 1056 consecutive FNA samples with indeterminate cytology, these authors showed that 87-95% of mutation positive samples with indeterminate cytology were malignant at histology (see Table **1** for sensitivity, specificity, negative and positive predictive values and accuracy for the different diagnostic categories) [57]. The false positive results were represented by 9 RAS mutation positive samples, which turned to be benign (follicular adenoma) on histological examination. The latter results are still acceptable since follicular adenomas carrying RAS mutation are considered premalignant lesions [57, 59]. Less acceptable is the relative high percentage of false negative results, ranging from 32% to 43% in the 3 different diagnostic categories with indeterminate cytology, responsible for the low sensitivity observed (Table **1**).

These results could have been expected since the proto-oncogene mutations analyzed are held responsible for about 60-70% of all DTC, while the remaining do not show recognized somatic gene alterations. The second approach, named gene-expression classifier (GEC), was intended to identify benign, rather than malignant, thyroid nodules with the main aim to reduce unnecessary thyroid surgery in patients with indeterminate cytology [60-62]. The GEC, called Afirma and developed by Veracyte Inc. (San Francisco, CA), consists of a microarray analyzing the expression of 167 different genes in the RNA extracted from FNA biopsies [60]. Alexander and colleagues in a prospective multicenter study validated the method on a case study of 265 nodules with indeterminate cytology [61]. As expected for a test designed to identify benign samples, the negative predictive value ranged between 85% and 95% in the three diagnostic categories with indeterminate cytology (see Table **1**). As for the proto-oncogene mutation test, a major caveat rising from the results of this study was that as many as 6 to 18% of nodules classified as benign by the GEC (false negative) were likely to harbor a malignant lesion [61]. To overcome these limitations it was also thought that combining a high specific genetic mutation test, such as the $BRAF^{V600E}$ one, with the highly sensitive GEC test, could lead to a better discrimination of malignant from benign lesions in nodules with indeterminate cytology [63]. The results of such attempt were recently reported by Kloos and colleagues, who analyzed 208 nodules with indeterminate cytology and found that the analysis of $BRAF^{V600E}$ did not improve the diagnostic accuracy of the GEC alone [64].

In conclusion, while the two diagnostic methods above described represent an improvement of the molecular diagnosis of thyroid nodules they still present important caveats which limit at the moment their clinical application. It is possible that in a near future the identification of additional genes involved in thyrocyte malignant transformation may improve the diagnostic accuracy of the proto-oncogene mutation test, as well as the implementation of the GEC with additional genes such as miRNAs, thought to play a role in cell transformation and cancer progression, may increase the test predictivity [54, 55, 65].

MOLECULAR DIAGNOSIS IN CERVICAL LYMPH NODES

As above mentioned, the accurate diagnosis of loco-regional lymph node metastasis is of primary importance for the initial surgical approach as well as for prognostic stratification and follow-up of thyroid cancer patients [15, 17-21, 66, 67]. The FNAC represents the gold standard technique for the diagnosis of CLN suspected to harbor metastatic disease from thyroid cancer as well as from other primary tumors [15, 17-21, 66]. The technique accuracy, highly dependent on the experience and

Table 1: Diagnostic Performance of the Proto-Oncogene Mutation and Gene-Expression Classifier Tests in the Diagnosis of Thyroid Nodules with Indeterminate Cytology

Test	Sensitivity	Specificity	NPV	PPV	Accuracy
Proto-oncogene mutations (ref. [56])					
Atypia/follicular lesion of undetermined significance	63%	99%	94%	88%	94%
Follicular /suspicious for follicular neoplams	57%	97%	86%	87%	86%
Suspicious for malignancy	68%	96%	72%	95%	81%
Gene-expression classifier (ref. [59])					
Atypia/follicular lesion of undetermined significance	90%	53%	95%	38%*	62%*
Follicular / suspicious for follicular neoplams	90%	49%	94%	37%*	59%*
Suspicious for malignancy	94%	52%	85%	76%*	78%*

NPV, negative predictive value; PPV, positive predictive value. *Calculated from data reported in reference [59].

ability of the cytopathologist, has been reported to vary from 73% to 94% [68-71]. A major limitation to FNAC is represented by the inadequate cellularity or non-representative sampling often associated with cystic lymph nodes which prevents diagnosis in about 20% of specimens [23, 24]. In order to solve this diagnostic caveat, in 1992 Pacini and colleagues first described the utility of thyroglobulin (Tg) protein measurement in the washout of the needle used for the FNAC [72]. In this initial study they reported that Tg detection in fine needle aspirates of non-thyroidal neck masses showed 100% sensitivity in detecting CLN metastasis during the follow-up of patients who were previously treated by total thyroidectomy and ^{131}I ablation, while FNAC alone showed a sensitivity of 85% [72]. Since then, different studies, measuring Tg protein and/or mRNA, confirmed the utility of Tg determinations in the detection of DTC metastasis in CLN [67-71, 73-81]. It is worth noting that, differently from serum Tg measurement, Tg detection in the washout of fine-needle aspirates from CLN is not affected by circulating anti-Tg antibodies present in about 20% of patients affected by DTC [82]. In addition, the same technique may be used to detect calcitonin (CT) protein and/or mRNA for the detection of metastatic medullary thyroid cancer (MTC) [81]. The diagnostic performance of this molecular approach, in terms of sensitivity, specificity, negative and positive predictive value and accuracy was shown to be excellent [67-82]. Based upon these pieces of evidence, the routine association of Tg protein detection with FNAC in the preoperative diagnosis of suspicious CLN was recommended [17, 18]. However, in a recent study, we showed that the diagnostic accuracy of Tg determination, although excellent, was not statistically superior to that of FNAC alone, nor the combination of the two tests improved the diagnostic accuracy of FNAC alone [81]. However, we found that Tg and CT detection in suspicious metastatic CLN was of clinical value in cases in which FNAC provided uninformative diagnosis, as often occur in presence of cystic lymph nodes, or when FNAC diagnosis is inconsistent with respect to patient's biochemical and/or clinical parameters [67, 81]. Following these observations we proposed that samples for Tg and CT mRNA and protein analysis from CLN suspicious for metastatic thyroid cancer should be always collected, but their measurements should be restricted to cases in which FNAB-C gives uninformative or inconsistent diagnosis with respect to patient's biochemical and/or clinical parameters (see the flow chart in Figure **1**).

It is also worth to mention that this approach may significantly reduce the costs of the management of patients with thyroid cancer whose incidence, as above described, has been increasing over the last years being actually the fifth most common cancer in women.

NEW EMERGING PROGNOSTIC MOLECULAR MARKERS IN THYROID CANCER PROGNOSIS

The most widely used staging system for thyroid cancer patients is represented by the TNM (Tumor size, lymph Node and distant Metastasis) system

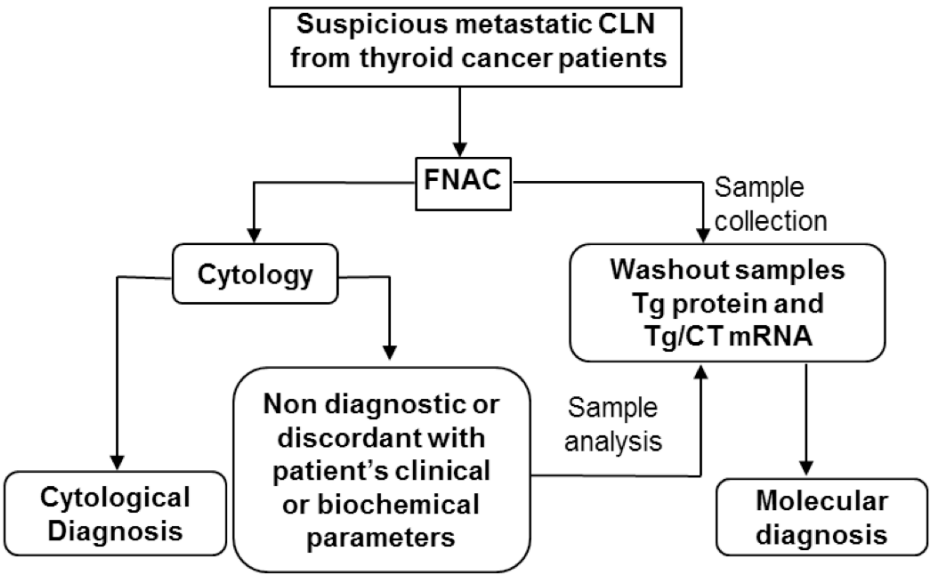

Figure 1: flow-chart for diagnosis of suspicious metastatic cervical lymph nodes (CLN) from thyroid cancer patients (adapted from BMC Clinical Pathology 2013;13:7). Following fine-needle aspiration cytology (FNAC) samples for Tg protein and Tg/CT mRNA measurements should be collected, but their analysis restricted to cases with uninformative or clinically unsound FNAC diagnosis. Tg, thyroglobulin; CT, calcitonin.

developed by the American Joint Committee on Cancer and the International Union Against Cancer [83]. This staging system is strongly influenced by the patient's age with those above 45 yr receiving a worse prognosis despite other factors being equal [83]. The TNM, as well as other staging systems proposed by recognized international organizations, are capable to make a rough prediction of the high or low risk of cancer mortality leaving, however, in the same risk group patients showing different disease-specific progression and survival time. Similarly, they fail to predict the risk of cancer recurrences [84]. Regarding the latter, both the European (ETA) and the American Thyroid Associations (ATA) proposed practical guidelines to estimate the risk of recurrences in which the TNM parameters are integrated by additional clinical features such as the tumor histological variant, the results of post-ablative whole body scan and the serum Tg levels [15, 18]. Despite that, patients included in the same risk group showed a very heterogeneous behavior in terms of disease-free interval. In addition, the stratification risk proposed by the ATA and the ETA is not accurate in predicting the long-term outcome in differentiated thyroid cancer patients, showing a very low positive predictive value [85]. As a consequence, the identification of new prognostic molecular biomarkers able to testify tumor aggressiveness is required.

In this context, $BRAF^{V600E}$ mutation, the most prevalent genetic alteration observed in 29-87% of PTC, received considerable attention as new prognostic marker in PTC [86, 87]. In fact, several reports showed the association of this mutation with factors related to poor prognosis, such as the presence of extrathyroidal extension, lymph node metastasis, advanced tumor stage, reduced disease-free interval and patient survival [86, 87]. However, despite the initial enthusiasm, a debate is ongoing about the clinical relevance of these findings [86, 87]. In particular, further studies failed to associate the $BRAF^{V600E}$ with poor prognosis in PTC patients [88-92]. In addition, the frequency of BRAF mutation in PTC is high (about 50%), compared with the poor outcomes (about 20%), and as a consequence a large percentage of patients would face the risk of over- or under-treatment based only on the analysis of the $BRAF^{V600E}$ mutation [87].

Over the last few years, our and other research groups investigated the prognostic value of the components of the urokinase plasminogen activating system (uPAS) in thyroid cancer patients [91, 93-108]. The uPAS consists of the urokinase plasminogen activator (uPA), its cognate cell membrane receptor (uPAR) and two main inhibitors, the plasminogen activator inhibitor-1 (PAI-1) and -2 (PAI-2) [109]. It controls several pathophysiological processes, including human cancer progression where induces extracellular matrix degradation, activation of latent growth factors, malignant cells proliferation and spread and tumor neo-angiogenesis [109, 110]. The prognostic value of uPAS components was validated for different cancer types and, as a rule, overexpression of one or more uPAS components was shown to associate with a higher risk of relapse and poor clinical outcome [109, 110]. This is particularly evident in breast cancer, in which uPA and PAI-1 were shown to be among the most potent prognostic factors described to date, with a predictive value stronger than those of patient age, tumor size, estrogen and progesterone receptors, HER-2/neu or p53 expression [111-115]. This evidence led the American Society of Clinical Oncology to include both uPA and PAI-1 among the recommended breast tumor markers for clinical use [116]. Also in PTC, over-expression of uPA and uPAR significantly associates with high-risk clinicopathological factors such as lymph node metastasis, higher TNM stage and lower disease-free interval [91, 93-95]. In addition, Horvatić Herceg and colleagues on a case study of patients with benign and malignant thyroid cancers, including PTC, FTC, MTC and ATC, showed that high expression of uPA and PAI-1 associated with tumor size, extrathyroidal invasion, lymph node and distant metastasis, and progression-free survival [96]. All together, the above findings warrant further investigation to confirm the potential role of uPAS components in thyroid cancer prognosis.

Very recently, two new molecular markers potentially useful for the prognosis of thyroid cancer patients were described [92, 117]. The first is represented by the estrogen receptor (ER) shown to possess a prognostic value in several cancer types including breast, ovarian and lung cancers [118-123]. In particular, an elevated level of ERα expression is associated with an improved survival in breast and ovarian cancer patients, but with reduced survival in lung cancer, while the low expression of ERβ associates with a poor survival in ovarian, breast and lung cancer patients [118-123]. In a recent study Heikkilä and colleagues demonstrated that the reduction of ERβ expression associates significantly with poor outcome in FTC patients [117].

The second one is represented by the miR-146b [92]. MicroRNA (miR) are small (19–25 nucleotides),

noncoding RNA molecules that behave as negative regulators of gene expression [124]. They regulate important cellular functions as proliferation and apoptosis and may act as tumor suppressor genes and oncogenes [124]. Mature miRs typically down-regulate gene expression post-transcriptionally by inhibiting mRNA translation or promoting mRNA degradation by annealing to complementary sequences in 3′ untranslated regions of their target mRNAs [124]. In thyroid cancer tissues different miRs were found up-regulated and a number of them were suggested to have a diagnostic utility [65, 125]. One of them, the miR-146b, was shown to associate in PTC with extrathyroidal invasion, advanced tumor stage, BRAF mutation and disease-free survival [92, 126].

In conclusion, a number of new molecular markers potentially capable to predict disease outcome in thyroid cancer patients is emerging. The reported evidence warrant for each of them further clinical investigation in larger prospective multicenter trials to definitely prove their ability to predict disease outcome. This is likely to ameliorate the actual staging system thus allowing a tailored therapy and follow-up to the single patient needs**.**

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