The dilemma of indeterminate thyroid cytology: how many markers are needed for a reliable diagnosis?

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Indeterminate cytology arises from the limit inherent in traditional cyto-morphological analysis. Numerous benign processes can cause subtle cellular or nuclear changes, sometimes indistinguishable from findings of well-differentiated carcinoma. Moreover, follicular carcinoma can be diagnosed only if the tumor's ability to penetrate capsular or vascular structures is documented, but such a property can only be assessed at final histology.

Although there is no unanimous consensus terminology for classifying indeterminate FNA cytology specimens and different Countries utilize various criteria and definitions, all of them agree that such specific cytological subgroup of lesions represents the "*gray zone*" of thyroid FNA cytology. They can be generically named as indeterminate, and include hyperplasic follicular lesions, follicular adenoma, Hürthle cells adenoma, Hürthle cells cancer and follicular variant of papillary carcinoma. The prevalence of malignancy is □20%, and this figure is rather low to recommend surgery for all of them. Following such indication, in fact, surgery has been done unnecessarily in □80% of these patients and an increase number of complications as well as a costs rise were observed. Therefore, many efforts have been made to further subdivide such category in risk subgroups, using clinical, radiologic, cytological and molecular parameters (1). However, no clinical, radiological, laboratory or molecular test, when considered alone, is sensitive and, at the same time, specific enough to reliably discriminate whether a suspicious nodule identified on FNA is benign or malignant. For this reason, the combination of several molecular markers was proposed to improve accuracy of cytology and to solve the diagnostic dilemma of the suspicious nodules, one of the most frustrating diagnostic experiences for the pathologists and endocrinologists. The increasing number of molecular markers as well as their possible combinations of different test methods is becoming an issue in the diagnosis of thyroid nodules with suspicious cytological features. In one study, the expression of up to 167 different genes was analyzed in the cytological samples and, in addition, the occurrence of BRAF mutations was considered. A specific algorithm to evaluate the results was developed by one Company (Gene Expression Classifier, Veracyte Afirma®) (2,3) In another study, a total of 13 gene mutations and 42 gene fusions, analyzed by next generation sequencing, were included in a mutation/fusion panel (Thyroseq Ver. 2.0) (4). In other studies, a combination of different methods was suggested. The analysis of a complex mutation/fusion panel plus Gene Expression Classifier of specific miRNAs was adopted by other Companies (Interpace Diagnostic ThyGenX®/ThyraMIR™) (5). As an improvement compared to other previous test-methods, in a recent study, the molecular analysis was performed on the same single, routinely prepared FNA smear, initially used to categorize the sample as indeterminate. The test, in fact, does not require an additional FNA specimen collection (Rosetta GX Reveal®, Rosetta Genomics Ltd, Rehovot, Israel) (6,7). In this test, starting from a total of initial screening of 2,000 miRNA probes, a final set

of 24 miRNAs that showed differential expression in benign and malignant tumors were selected, validated and included in the diagnostic assay. We recently performed a comparative analysis on the different test-methods proposed for the same reason (8). Some of the test-methods considered are highly specific and they can be considered as excellent "rule-in" indicators. In particular, the best rule-in test is the BRAF mutation analysis. Occurrence of this mutation is always associated with thyroid malignancy, but unfortunately it is mostly associated with papillary types of tumors, easy to be recognized at cytology, and it showed one of the lowest sensitivity observed, with very high false negative rate. Conversely, there is no other test available with such comparable absolute diagnostic efficiency when we specifically look for its ability to exclude malignancy. In other words, no single good "rule-out" tests can be efficiently recommended to fulfill such goal. Many of these test-methods require shipping samples overseas to centralized laboratories that have developed in-house "laboratory-developed tests." Therefore, there is need for an *in vitro* diagnostic (IVD) test for local reference clinical laboratories or, hopefully, for a validated diagnostic *in vitro* kit. The issue is highly relevant and there are many unanswered questions yet. How many markers are needed to obtain a reliable diagnosis? In addition, besides the need for an expert cyto-pathologist, what are the other expertise, competence and instruments that are requested to render a correct diagnosis to our patients affected by a suspicious thyroid nodule? Should all the thyroidologists be trained to become expert molecular biologists? And above all, what is the maximum cost that can be charged to the patient and eventually reimbursed by the National Public Health System to receive an accurate and predictive diagnosis? In term of cost it is reasonable to expect that the introduction of the new molecular test-methods would result in a reduction in the costs because many patients will not be treated with surgery. However, inclusion of many different molecular markers and combining different test-methods all together would result in a dramatic increase of the cost. Many attempts have been made in the past to limit the number of molecular marker to be tested was previously performed and a test based on as few as only 3 genes was considered both feasible and promising for the cytological diagnosis of indeterminate thyroid nodules (9). Nevertheless, the trend is still to add molecular markers to increase statistical performances.

In the work presented by González *et al.* (10) the Authors tried to limit the number of molecular markers. They took advantage of the previous experience in other tumor tissues,

and in particular in breast cancer. So far, there are many different commercially available multigene marker panels, including genomic profiles and gene-expression assays of breast cancer specimens to evaluate the gene signatures of each single tumor and to use such information for both diagnostic and prognostic purposes. Such test-methods are based on the results of the expression analysis of a different set of genes (21 in the Oncotype DX, 50 in the Prosigna, 70 in the MammaPrint, 2 plus 5 in the Breast Cancer Index, 8 in the EndoPredict and 97 in the Genomic Grade Index) [for a review see (11)]. Among these tests the EndoPredict® assay, (Myriad Genetics, Salt Lake City, Utah, USA.) is in IVD format, can be routinely performed in a decentralized molecular pathology laboratory and can be applied with good reproducibility and accuracy also on FFPE tumor tissue specimens (12). Following such example, the authors analyzed a wide list of genes reported to show differential expression and/or biological significance in thyroid carcinogenesis/inflammation. Using qPCR and a linear discriminant analysis (LDA), they set up a novel multiplexed-10-gene qPCR thyroid genetic classifier (TGC), with high sensitivity and specificity, that can be performed as an IVD assay in reference laboratories. This study confirms that no single gene, when considered alone, reached the optimal statistical performance to properly classify the thyroid nodules as malignant or benign but there is need to combine at least 10 of them to obtain a good efficiency. González and co-workers have provided a good starting point to identify prior to surgery such difficult cancers using a prototype IVD assay. The clinical performance of this assay could be further improved in future studies, and will be further validated in the two ongoing independent multicenter clinical validity trials in a large set of indeterminate samples, announced by the Authors. The attempt to reduce the number of different molecular markers to be analyzed and, hence, the cost of the test is relevant and so the effort to set up a standardized IVD test. It is important to note that, before any method will be used in routine practice, a medico-economic assessment should be undertaken to compare the cost of the method itself with the benefits afforded by decreasing the number of unnecessary surgical procedures. Such analysis should be performed in any different socio-economic context and it is not easily applicable, "*tout court*", in any Country. Cost-effectiveness analysis performed in high-income Countries where insurance companies may cover the cost for expensive molecular tests may not be valid in low-income Countries, where such cost would be totally

charged to the patient. In a low-income Country like Italy, the cost of a single molecular test (3,000–5,000 USD) would be the same than that (3,300 EUR) reimbursed by the Public Health System for one thyroidectomy (excluding any complication). In such situation one would be persuaded to choose for thyroidectomy instead. The optimal test to be applied in any clinical context should be cheap and easy to be performed as a screening tool. One available test with such features is the Galectin-3 ThyroTest, based on the evaluation by immunocyto-histochemistry of the expression pattern of Galectin-3 on FNA-derived cellblock substrates (13,14). To increase the diagnostic ability of FNA in indeterminate nodules it would be ideal to combine low-cost test with as few as possible selected number of molecular markers and at the same time to limit the cost of the test to make them competitive with surgical procedures.

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Footnote

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Conflicts of Interest: A Bartolazzi is co-owner of an Italian Patent N. RM2008A000097 on the use of radiolabeled mAbs to Galectin-3 for thyroid cancer diagnosis. Another author has no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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