Impact of Proto-Oncogene Mutation Detection in Cytological Specimens from Thyroid Nodules Improves the Diagnostic Accuracy of Cytology

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Context: Fine-needle aspiration cytology (FNAC) is the gold standard for the differential diagnosis of thyroid nodules but has the limitation of inadequate sampling or indeterminate lesions.

Objective: We aimed to verify whether search of thyroid cancer-associated protooncogene mutations in cytological samples may improve the diagnostic accuracy of FNAC.

Study Design: One hundred seventy-four consecutive patients undergoing thyroid surgery were submitted to FNAC (on 235 thyroid nodules) that was used for cytology and molecular analysis of BRAF, RAS, RET, TRK, and PPRγ mutations. At surgery these nodules were sampled to perform the same molecular testing.

Results: Mutations were found in 67 of 235 (28.5%) cytological samples. Of the 67 mutated samples, 23 (34.3%) were mutated by RAS, 33 (49.3%) by BRAF, and 11 (16.4%) by RET/PTC. In 88.2% of the cases, the mutation was confirmed in tissue sample. The presence of mutations at cytology was associated with cancer 91.1% of the times and follicular adenoma 8.9% of the time. BRAF or RET/PTC mutations were always associated with cancer, whereas RAS mutations were mainly associated with cancer (74%) but also follicular adenoma (26%). The diagnostic performance of molecular analysis was superior to that of traditional cytology, with better sensitivity and specificity, and the combination of the two techniques further contributed to improve the total accuracy (93.2%), compared with molecular analysis (90.2%) or traditional cytology (83.0%).

Conclusions: Our findings demonstrate that molecular analysis of cytological specimens is feasible and that its results in combination with cytology improves the diagnostic performance of traditional cytology. **(*J Clin Endocrinol Metab* 95: 1365–1369, 2010)**

Fine-needle aspiration cytology (FNAC) is the gold standard for the diagnosis of thyroid nodules (1, 2) despite limitations related to inadequate or indeterminate samples. The discovery of genetic alterations in differentiated thyroid cancer prompted the search of somatic mutations in material obtained by fine-needle aspiration, aimed to increase the diagnostic accuracy of traditional cytology. Search of BRAF mutations alone or in combination with other oncogenes (RAS, BRAF, RET/PTC) in cytological material has been reported with encouraging results (3–14).

We aimed to evaluate the diagnostic utility of screening cytological samples of thyroid nodules undergoing surgery by searching a complete panel of mutations, those

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Abbreviations: DHPLC, Denaturing HPLC; FNAC, fine-needle aspiration cytology; NPV, negative predictive value; PPV, positive predictive value; PTC, papillary thyroid cancer.

associated with BRAF, RET, RAS, TRK, and PPRγ protooncogenes. Experiments were performed in 235 cytological specimens and the corresponding tumoral tissues taken at surgery.

Patients and Methods

We studied 174 consecutive patients (138 females) undergoing thyroid surgery. Mean age was 51.2 ± 13.8 yr (range 20–83 yr). The indication for surgery was an FNAC indicative (n = 48) or suspicious (n = 22) of thyroid cancer in 70 patients (40.2%), indeterminate lesion in 50 (28.7%), and presence of compressive symptoms in 54 (31.1%) patients with nodular goiter with benign or inadequate cytology but no suspicion of thyroid cancer. Before surgery, all patients repeated an FNAC (235 nodules) under ultrasound guidance (15) that was used for classical cytology (two thirds of the material) and molecular analysis (the remaining part). At surgery a sample of the nodules was collected for molecular analysis. All patients provided informed consent for both traditional cytology and molecular analysis.

Samples were screened for the presence of BRAF point mutations (V600E and K601E); H-K-NRAS point mutations at codons 12, 13, and 61; and RET/PTC rearrangements. TRK and PAX8-PPARγ rearrangements were searched only in samples negative for BRAF, RET, and RAS mutations.

DNA and RNA isolation from fine-needle aspiration

DNA was extracted using the QIAamp DNA microkit (QIAGEN, Valencia, CA) following kit instructions. RNA was extracted using SV total RNA isolation system (Promega, Madison, WI) and reverse transcribed into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Every sample was processed to verify the presence of thyroid cells by amplifying for thyroglobulin gene.

Search for BRAF and RAS point mutations in cytological material

For BRAF point mutations (V600E and K601E), exons 11 and 15 were amplified in a mixture containing 2× PCR master mix (AmpliTaq Gold PCR master mix; Applied Biosystems) and a final primer concentration of 200 nM at 52.5 C. To identify RAS mutations (H-, K-, and N-RAS), codons 12, 13, and 61 were amplified with 200 nM primer final concentration at 64.9 C for H-RAS and 58 C for K-RAS and N-RAS.

RNA isolation and RT-PCR from frozen thyroid tissues

Tissues were collected at surgery into a 1.5-ml microcentrifuge tube containing Allprotect tissue reagent (QIAGEN). Total RNA was extracted and reverse transcribed into cDNA. For every sample, 1 μg of cDNA was amplified in mixture containing the 2× PCR master mix and a final primer concentration of 200 nM.

Detection of TRK, RET/PTC, and PAX8-PPARγ rearrangements

TRK and RET/PTC rearrangements were searched with primary and nested PCR amplification as previously reported (10, 16). Nested PCR was also performed to detect PAX8-PPARγ rearrangement (16). For RET/PTC analysis, Southern blot technique was also performed as previously described (11).

Denaturing HPLC (DHPLC) and sequencing

PCR products were analyzed with DHPLC technique at specific temperatures to confirm the presence/absence of mutations. Positive samples were subjected to direct sequencing (Primm, Milan, Italy).

Results

Oncogene mutations

Analysis of BRAF (V600E and K601E) and N-, K-, H-RAS was possible in all 235 cytological and tissue samples. Search for RET/PTC rearrangement (RET/PTC1, RET/PTC3, RET/PTCX) was possible in all tissues but in only 49% of cytological samples (113 of 235) due to low quantity of cytological material (n = 84) or bad quality of the extracted RNA (n = 38).

Mutations were found in 67 of 235 cytological samples (28.5%). The most frequent mutations was BRAF (33 of 235, 14.0%), followed by RAS mutations (23 of 235, 9.8%) and RET/PTC rearrangements (11 of 235, 4.7%). Considering only the 67 mutated nodules, BRAF (V600E) point mutations were present in 33 of 67 (49.3%), RAS point mutations in 23 (34.3%), and RET/PTC rearrangements in 11 of 67 (16.4%). The presence of two mutations was found in four cases (BRAF and RAS in three and H- and N-RAS mutations in one). In tissue samples we found 76 mutations (32.3%), including 35 BRAF (V600E) (14.9%), 25 RAS (10.6%), and 16 RET/PTC (6.8%). A double mutation was found in four cases, the same found in cytological samples. Thus, in 88.2% of the cases, the mutation found in cytological material was confirmed in the tissue sample. The 11.8% discrepant results were represented by mutations in the tissue sample not found in the cytological sample. The discrepancy was more frequent in cases of RET/PTC rearrangements and, in cases of RAS, was mainly due to low sensitivity of DHPLC on cytology rather than direct sequencing.

Comparison of cytology and molecular biology

The results of classical cytology, mutation analysis on cytological samples and final histology are detailed in Fig. 1. In the category of suspicious thyroid cancer (n = 54), 37 samples (68.5%) harbored a mutation (21 BRAF, six RET-PTC, 10 RAS), and all of them were papillary thyroid cancer (PTC) at final histology. Of the 17 patients without mutations, nine were PTC, four were follicular adenomas and four were hyperplastic nodules.

Among the 87 benign cytologies, nine (10.3%) carried a mutation (two BRAF, two RET-PTC, and five RAS); six

Cytology (n=235)	Mutation (in cytology)	Histology
Suspicious for Thyroid cancer (n=54)	BRAF 21	PTC 21
	RET/PTC 6	PTC 6
	RAS 10	PTC 10
	None 17	PTC 9 FA 4 Hyperplastic 4
Benign (87)	BRAF 2	PTC 2
	RET/PTC 2	PTC 2
	RAS 5	PTC 2 FA 3
	None 78	PTC 2 FTC 1 FA 10 Hyperplastic 65
Indeterminate (n=41)	BRAF 2	PTC 2
	RET/PTC 2	PTC 2
	RAS 3	PTC 2 FA 1
	None 34	PTC 1 FA 25 Hyperplastic 8
Inadequate (n=53)	BRAF 8	PTC 8
	RET/PTC 1	PTC 1
	RAS 5	PTC 2 HCC 1 FA 2
	None 39	PTC 2 FTC 2 FA 11 Hyperplastic 24

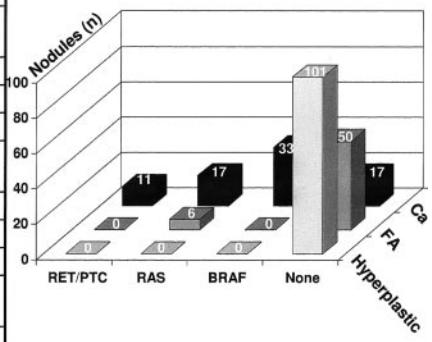

FIG. 1. Correlation between results of cytology, molecular biology on cytological samples, and final histology. FTC, Follicular thyroid cancer; HCC, Hürthle cell cancer; FA, follicular adenoma; Ca, cancer. The graph on the *right* shows the distribution of RET/PTC, RAS, and BRAF mutations according to final histology.

were PTC and three were follicular adenomas (all with RAS mutation). Of the 78 samples with no mutations, two were PTC and one was follicular thyroid cancer, 10 were follicular adenomas, and 65 were hyperplastic nodules.

In the group of indeterminate lesions (n = 41), seven samples (17%) were mutated (two BRAF, two RET-PTC, three RAS). At final histology, all but one (follicular adenoma) were PTC. Of the 34 samples with no mutation, 25 were follicular adenomas, eight were hyperplastic nodules, and only one was PTC.

In the group of inadequate cytology (n = 53), 14 samples (26.4%) were mutated (eight BRAF, five RAS, one RET/PTC). Eleven were PTC, one was Hürthle cell cancer, and the other two were follicular adenomas. Of 39 inadequate samples with no mutations, four were cancers (two PTC, two follicular thyroid carcinoma), 11 were follicular adenomas, and 24 were hyperplastic nodules.

No TRK or PAX8/PPRγ rearrangements were found in cytological or tissue samples screened for these oncogenes.

A total of 78 cancers was found at final histology. As shown in the graphic of Fig. 1, molecular biology detected 61 of them (78.2%), whereas traditional cytology correctly diagnosed 46 of them (58.9%). Among 56 follicular adenomas, six (10.7%) carried RAS mutations. No mutation was associated with a final diagnosis of hyperplastic nodule (n = 101).

In summary, the presence of any mutation at cytology was associated with a final diagnosis of cancer 91.1% of the times and with a diagnosis of follicular adenomas in 8.9%.

BRAF or RET/PTC mutations were always associated with cancer, whereas RAS mutations were mainly associated with thyroid cancer (74%) but also with follicular adenomas (26% of the time). No mutations were associated with cancer 10.1% of the time and follicular adenomas or hyperplastic nodules in the remaining cases (89.9%).

As shown in Table 1, traditional cytology had good specificity (94.9%) but low sensitivity (59.0%), with positive predictive value (PPV) and negative predictive value (NPV) of 85.2 and 82.3%, respectively. Molecular analysis, considering follicular adenomas with RAS mutation as false-positive results, had similar good specificity (96.2%) but better sensitivity (78.2%) and better PPV and NPV (91.0 and 89.9%, respectively). The combination of the two techniques further improved the sensitivity (89.7%) and NPV (94.9%), giving the best total accuracy (93.2%), compared with molecular analysis alone (90.2%) or traditional cytology alone (83.0%). If we considered follicular adenomas with RAS mutations as true positive (assuming that they are preneoplastic lesions if not already min-

TABLE 1. Diagnostic performance of cytology, molecular analysis, or a combination of both

Diagnostic modality	Sensivity TP/TP+FN (%)	Specificity TN/FP+TN (%)	PPV TP/TP+FP (%)	NPV TN/TN+FN (%)	Accuracy TP+TN/All (%)
Cytology (positive for malignancy)	59.0	94.9	85.2	82.3	83.0
Molecular analysis (mutation in malignancy)[a]	78.2	96.2	91.0	89.9	90.2
Molecular analysis (mutation in malignancy)[b]	79.8	100	100	89.9	92.8
Cytology and molecular analysis[a]	89.7	94.9	89.7	94.9	93.2
Cytology and molecular analysis[b]	90.5	98.7	97.4	94.9	95.7

TP, True positive; TN, true negative; FN, false negative; FP, false positive.

[a] Mutated follicular adenomas computed as false positive.

[b] Mutated follicular adenomas computed as true positive.

imal cancers), molecular analysis had a sensitivity of 79.8% and specificity of 100%. The combination of the two techniques further improved the total accuracy (95.7%), compared with molecular analysis alone (92.8%) or traditional cytology alone (83.0%).

Discussion

Our study has the strength of being a surgical series, thus including also documented benign lesions, of screening for a complete panel of protooncogene mutations and having the comparison with the results in tissue samples. In nearly half of our cytological samples, we could not assess RET/PTC rearrangements due to low or bad quality of RNA. This is not surprising because we performed molecular analysis in the same routine conditions used for traditional cytology (one or two needle passes). This problem may be overcome by increasing the needle passes, as suggested (14, 17). Nevertheless, the overall concordance was satisfactory (88.2%), and the discrepant results in favor of tissue samples (11.8%) were mainly dependent from RET/PTC analysis.

Molecular biology on cytological samples correctly identified 78.2% of the thyroid cancers as opposed to 58.9% identified by traditional cytology. As in other series (3, 10–14), BRAF mutations or RET/PTC rearrangements were found only in cancer samples and never in benign nodules or follicular adenomas. RET/PTC mutations were not restricted to RET/PTC-1 and -3, indicating the need of searching for any RET/PTC rearrangement.

As in the study by Nikiforov *et al.* (14), RAS was mutated in 21.8% of thyroid cancers and 10% of the follicular adenomas but never in hyperplastic nodules. Theoretically when the RAS mutation is found in follicular adenoma, it should be considered as a false-positive result. However, our data are too preliminary to advocate total thyroidectomy in this category, and for the time being, we should probably behave as we use to do based on cytology only (*i.e.* lobectomy with or without frozen sections).

In the analysis of diagnostic performance, the overall PPV increases from 91 to 100% when the RAS mutation is computed as false positive or true positive, respectively. An FNAC indicative of thyroid cancer is very rarely falsely positive. In this category molecular biology adds little diagnostic information, although the knowledge of a mutation associated with worst outcome (BRAF) may influence the surgical strategy (18, 19). The great advantage of molecular analysis is found in indeterminate, benign, and inadequate lesions. In these groups, molecular biology was able to detect 24 of 32 thyroid cancers missed by traditional cytology.

Molecular analysis of the indeterminate group has a particular clinical utility because it allows the precise prediction of malignant cases to be submitted immediately to total thyroidectomy instead of diagnostic lobectomy carrying the risk of completion thyroidectomy when the final histology is cancer.

In conclusion, our results confirm and extend previous studies demonstrating that molecular analysis on cytological specimens is feasible and that, when including the analysis of a complete panel of protooncogene, its result significantly increases the diagnostic accuracy of traditional cytology.

In practice, we suggest total thyroidectomy whenever cytology is indicative of cancer and/or a mutation is found. When no mutation is detected, the therapeutic strategy should be dictated by the clinical picture and the results of FNAC: follow-up for benign cytology, repeat FNAC for inadequate samples, and lobectomy for indeterminate lesions.

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