Thyroid

Relevance of BRAFV600E mutation testing versus RAS point mutations and RET/PTC rearrangements evaluation in the diagnosis of thyroid cancer

Journal:	*Thyroid*
Manuscript ID:	THY-2014-0338.R2
Manuscript Type:	Clinical or Basic Original Study
Date Submitted by the Author:	n/a
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Keyword:	Thyroid Cancer- clinical, Thyroid Nodules, Molecular Biology
Abstract:	Background: A molecular profile including BRAF and RAS mutations as well as RET/PTC rearrangement evaluation has been proposed to provide an accurate pre-surgical assessment of thyroid nodules and to reduce the number of unnecessary diagnostic surgeries, sparing patient's health and saving healthcare resources. However, the application of such molecular analyses may provide different results among different centers and populations in real life settings. Our aim was to evaluate the diagnostic utility of assessing the presence of BRAF and RAS mutations and RET/PTC1 and RET/PTC3 rearrangements in all cytological categories in an Italian group of thyroid nodule patients assessed prospectively and to understand whether and which mutation testing might be helpful in cytologically indeterminate nodules.

Methods: 911 patients were submitted to ultrasound and fine needle aspiration biopsy examination. Cytological evaluation was performed in parallel with molecular testing and compared to pathological results in 940 thyroid nodules, including 140 indeterminate lesions.

Results: BRAF mutation testing provided the best contribution to cancer diagnosis, allowing to detect the disease at an early stage and to identify indeterminate nodules in which diagnostic lobectomy could be spared. On the contrary, RAS and RET/PTC analysis did not further increase diagnostic sensitivity for thyroid cancer. In addition, we found RET/PTC rearrangements in benign lesions, indicating that this molecular marker might not be useful to detect thyroid cancer.

Conclusion: BRAFV600E mutation analysis is superior to RAS point mutations and RET/PTC rearrangements evaluation in the diagnosis of thyroid cancer even in indeterminate lesions.

1 **Relevance of *BRAFV600E* mutation testing versus *RAS* point mutations and *RET/PTC***

2 **rearrangements evaluation in the diagnosis of thyroid cancer**

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27 Running title: *BRAF* is superior to *RAS* and *RET/PTC* in PTC diagnosis

28 Key words: *BRAF V600E* mutation, *RAS* mutations, *RET/PTC* rearrangements, papillary thyroid

29 cancer, diagnosis

30

31 **ABSTRACT**

32 **Background:** A molecular profile including *BRAF* and *RAS* mutations as well as *RET/PTC*

33 rearrangement evaluation has been proposed to provide an accurate pre-surgical assessment of

34 thyroid nodules and to reduce the number of unnecessary diagnostic surgeries, sparing patient's

35 health and saving healthcare resources. However, the application of such molecular analyses may

36 provide different results among different centers and populations in real life settings. Our aim was

37 to evaluate the diagnostic utility of assessing the presence of *BRAF* and *RAS* mutations and

38 *RET/PTC1* and *RET/PTC3* rearrangements in all cytological categories in an Italian group of

39 thyroid nodule patients assessed prospectively and to understand whether and which mutation

40 testing might be helpful in cytologically indeterminate nodules.

41 **Methods:** 911 patients were submitted to ultrasound and fine needle aspiration biopsy

42 examination. Cytological evaluation was performed in parallel with molecular testing and

43 compared to pathological results in 940 thyroid nodules, including 140 indeterminate lesions.

44 **Results:** *BRAF* mutation testing provided the best contribution to cancer diagnosis, allowing to

45 detect the disease at an early stage and to identify indeterminate nodules in which diagnostic

46 lobectomy could be spared. On the contrary, *RAS* and *RET/PTC* analysis did not further increase

47 diagnostic sensitivity for thyroid cancer. In addition, we found *RET/PTC* rearrangements in benign

48 lesions, indicating that this molecular marker might not be useful to detect thyroid cancer.

49 **Conclusion:** *BRAFV600E* mutation analysis is superior to *RAS* point mutations and *RET/PTC*

50 rearrangements evaluation in the diagnosis of thyroid cancer even in indeterminate lesions.

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52 **Abtract word count:** 243

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53 **INTRODUCTION**

54 The diagnostic and therapeutic approach to thyroid cancer has been highly debated in the last

55 years. Ultrasound (US), cytology and molecular profiling (by mRNA gene expression platforms,

56 protein immunocytochemistry, miRNA panels, and by screening for somatic mutations including

57 *BRAFV600E* and *RAS* mutations as well as *RET/PTC1, RET/PTC3, PAX8/PPARγ, TK* and *ALK*

58 rearrangements) have been employed in order to provide the most accurate pre-surgical

59 assessment of thyroid nodules with the aim of increasing the sensitivity for cancer detection and of

60 avoiding surgery for lesions erroneously identified as malignant (1, 2, 3). The availability of pre-

61 surgical information improved preoperative risk stratification and often influenced the extent of

62 surgery (4, 5, 6, 7). The revised American Thyroid Association (ATA) guidelines indicate that

63 thyroid cancer should be treated according to risk stratification, assessed on the basis of disease

64 stage (8). The provided evidence indicates that treatment needs to be tailored according to the risk

65 of recurrence, suggesting that a more conservative attitude, avoiding radioiodine ablation, may be

66 indicated for patients with very low risk of recurrence (9, 10). As a consequence, early diagnosis

67 is crucial in order to detect the disease at an early stage and to guide the patient to a less

68 aggressive treatment thereby avoiding unnecessary risks for the patient's health and saving

69 healthcare resources (11, 12). The main diagnostic tool consists in fine needle aspiration biopsy

70 (FNAB), which, however, cannot provide a definitive diagnosis in cases with non diagnostic (ND)

71 or indeterminate cytology. The latter may represent a malignant lesion in ~20% of the cases, that

72 are not accurately predictable by ultrasound (US) risk factors and thus lead to the need for

73 diagnostic surgery (13). The preoperative use of molecular markers is still highly debated, among

74 other reasons because the incidence of mutations in the different categories outlined in the

75 Bethesda System for Reporting Thyroid Cytopathology (BSRTC) (14) is still unknown. To date,

76 the ATA guidelines suggest considering molecular testing only to refine a cytological

77 indeterminate result (8). Moreover, genetic, environmental and clinical background may

78 profoundly impact the incidence of mutations and hence there is a need to explore the applicability

3

79   of molecular testing of thyroid nodules in different populations in the clinical setting. The aim of

80   our study was to evaluate the diagnostic utility of assessing the presence of three previously

81   employed thyroid cancer molecular markers, including *BRAF* and *RAS* mutations, as well as

82   *RET/PTC1* and *RET/PTC3* rearrangements, in FNAB material from all cytological categories in a

83   "real life" context involving an Italian group of thyroid nodule patients, in order to improve

84   patient management and surgical treatment. In addition, we aim to assess mutation incidence in

85   each Bethesda category and to understand whether and which mutation testing might be helpful in

86   indeterminate nodules.

87   We therefore assessed the feasibility to obtain reliable results from FNAB material for the search

88   for these molecular markers (*BRAF V600E, RAS* mutations, *RET/PTC* rearrangements) in daily

89   clinical practice employing previously reported methods with slight modifications.

90

91   **MATERIALS AND METHODS**

92   Subjects

93   From January 2007 to July 2013, a total of 6500 thyroid nodules from 5800 patients were

94   submitted to FNAB procedure at the Section of Endocrinology of the University of Ferrara.

95   Among these, 940 FNAB specimens from 911 consecutive patients, displaying at least 2 clinical

96   and/or US characteristics of suspected malignancy, prospectively underwent the evaluation for

97   somatic mutations, including *BRAF V600E* and *RAS* point mutations and *RET/PTC1* and

98   *RET/PTC3* rearrangements, a panel partially overlapping the approach described previously by

99   Nikiforov et al. (15). Patients gave written informed consent for molecular analysis and data

100   collection.

101

102   Medical and US examination

103   All 911 patients recruited in this study were submitted to a careful US examination by a single

104   operator (S.L.) during routine medical care. The collected US features included nodule size (<or

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105 >1 cm), structure (solid, mixed, or cystic), echogenicity (iso-, hypo-, or hyperechoic), presence or

106 absence of micro calcifications, and margins. In addition, the patients' clinical information

107 regarding age, sex, family history of thyroid cancer or history of previous external beam radiation

108 exposure was collected.

109

110 FNAB procedures

111 All 940 US-guided FNAB procedures were performed by two experienced endocrinologists (G.T

112 and P.F.) using a standardized protocol, as previously described (16). Cytological evaluation was

113 performed in parallel with molecular testing. All FNAB results were categorized according to the

114 BSRTC (14), including class III (atypia of undetermined significance/follicular lesion of

115 undetermined significance: AUS/FLUS), IV (follicular neoplasm or suspicious for a follicular

116 neoplasm: FN) and V (suspicious of malignancy: SM) categories in the group of indeterminate

117 lesions.

118

119 DNA and RNA isolation

120 FNAB material from a needle pass through the nodule was used for cytology (performed at the

121 Section of Pathology of the University of Ferrara) and a second pass was collected in 5 ml of RNA

122 Later solution (Resnova) for molecular analysis, performed at the Laboratory of the Section of

123 Endocrinology of the University of Ferrara. Genomic DNA for *BRAF* and *RAS* somatic mutation

124 analysis was obtained as previously described (16, 17). Total RNA isolation for *RET/PTC1* and

125 *RET/PTC3* rearrangements evaluation was performed by centrifuging 2 ml of FNAB sample for 5

126 minutes at 5000 x g and the pellet was suspended in 350 µl of RLT Lysis Buffer (Qiagen, Hilden,

127 Germany). Later, the samples were processed in the QIAcube instrument (Qiagen) using the

128 RNeasy micro kit (Qiagen) according to manufacturers protocol, obtaining 30 µl of purified total

129 RNA. Samples were then processed as described in the following paragraphs. All samples

130 displaying a genetic variation were tested in a second assay by a different technician.

5

131   <u>*BRAF* and *RAS* mutation analysis</u>

132   *BRAFV600E* mutation analysis was performed as previously described (16, 17), employing a well

133   established methodology.

134   A first evaluation of *RAS* mutations was performed by applying Real Time Polymerase Chain

135   Reaction amplification followed by High Resolution Melting (HRM) analysis. Amplification of

136   *RAS* gene targets (codon 12, 13 and 61 of *N-RAS*, *H-RAS* and *K-RAS* gene isoforms) was

137   performed by using the MeltDoctor HRM Mastermix (Life Technologies, Carlsbad, CA, USA)

138   and specific primers (*N-RAS* exon 2 FOR 5' – TTGCTGGTGTGAAATGACTGAGT – 3' and

139   REV   5' –   TAGCTGGATTGTCAGTGCGC – 3';   *N-RAS*   exon   3   FOR: 5'   –

140   CAGAAAACAAGTGGTTATAGATGGTGA   –   3'   and   REV   5'   –

141   CAAATACACAGAGGAAGCCTTCG   –   3';   *H-RAS*   exon   2   FOR: 5'   –

142   GGAGCGATGACGGAATATAAGC – 3' and REV 5' – GTATTCGTCCACAAAATGGTTCTG

143   – 3'; *H-RAS* exon 3 FOR 5' – GGAAGCAGGTGGTCATTGATG – 3' and REV 5' –

144   GCATGTACTGGTCCCGCAT   –   3';   *K-RAS*   exon   2:   FOR   5'   –

145   TCACATTTTCATTATTTTTATTATAAGGC   –   3'   and   REV   5'   –   GA

146   TTCTGAATTAGCTGTATCGTCAAG   –   3';   *K-RAS*   exon   3:   FOR   5'   –

147   TCCAGACTGTGTTTCTCCCTTC – 3' and REV 5' – TACACAAAGAAAGCCCTCCC – 3').

148   Mutated samples were then genotyped by direct sequencing using the same primers on the 3130

149   Genetic Analizer (Life Technologies) employing the Ready Reaction Cycle Sequencing 1.1 mix

150   (Life Technologies). This approach, which is very similar to that previously employed (18),

151   allowed to obtain reliable results from FNAB material with a turn-around time of 72 hours.

152

153   <u>RET/PTC rearrangement analysis</u>

154   For the evaluation of *RET/PTC1* and *RET/PTC3* rearrangements, total RNA from FNAB samples

155   was analyzed by One Step Real Time RT-PCR, performed on a 7900 HT Real Time System (Life

156   Technologies, Carlsbad, CA USA), employing a modified method as compared to Nikiforov et al.

6

157 (15). The presence of *RET/PTC1* and *RET/PTC3* rearrangements has been assessed using two

158 different custom Taqman Gene Expression assays (Life Technologies), each represented by a

159 rearrangement specific primer-probe set; probes have been designed centred on the rearrangement

160 site, in order to avoid false positive results. Sequences of primers and probes for *RET/PTC1* were:

161 FOR: 5'- CGCGACCTGCGCAAA – 3', REV 5 – CAAGTTCTTCCGAGGGAATTCC – 3', and

162 PROBE: 5' - FAM-CCAGCGTGACCATCGAGGATCCAAAGT-NFQ – 3'. Sequences of

163 primers and probes for RET/PTC3 were: FOR: 5' – CCCCAGGACTGGCTTACCC – 3', REV 5'

164 – CAAGTTCTTCCGAGGGAATTCC – 3' and PROBE: 5' – FAM-

165 AAAGCAGACCTTGGAGAACAGTCAGGAGG-NFQ – 3'. All runs were multiplexed with

166 Eukaryotic 18S rRNA Endogenous Control (Life Technologies). The reaction mix included iScript

167 One-Step RT-PCR Kit for probes (Bio-Rad, Hercules, CA USA) and the appropriate Taqman

168 assays, described above. To test the method sensitivity, each target sequence assay was diluted

169 1:10, 1:100, 1:1000 and 1:10000 in not-rearranged cDNA. Both rearrangements were correctly

170 identified up to a 1:1000 dilution by the employed method. To exclude the possibility of

171 crossreactions, *RET/PTC1* and *RET/PTC3* assays were employed to amplify *RET/PTC3* and

172 *RET/PTC1* targets respectively, and no signal was obtained. RNA from one or more tumors or cell

173 lines known to carry a particular rearrangement was used as a positive control. This approach

174 allowed obtaining reliable results from FNAB material with a turn-around time of 24 hours.

175

176 <u>Statistical analysis</u>

177 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were

178 calculated for each detection method and for combined methods, considering histology as the gold

179 standard. Statistical analysis was carried out using the R Software package 3.0.2 (R Foundation

180 for Statistical Computing, Vienna, Austria). The chi square test (with Yates continuity correction)

181 was employed to compare the diagnostic sensitivity of cytology with that observed performing

7

182 both cytology and genetic analysis and to assess the presence of a significant association between

183 the presence of each mutation and US features. A $p < 0.05$ was considered significant in all tests.

184

185 **RESULTS**

186 <u>Patient findings</u>

187 Among the 911 patients who participated in the study, 51 had a family history of thyroid cancer,

188 712 were female and 199 males, with a mean age of 59 ± 0.46 years (age range $25 - 81$ years).

189 Patients with BSRTC class V and VI lesions, or with a nodule displaying *BRAF V600E* mutation

190 (independently of cytology results), or with large goiters underwent total thyroidectomy (TT).

191 Patients with repeatedly class I cytology and patients with BSRTC class IV lesions, or with a

192 nodule displaying either *RAS* mutations or *RET/PTC* rearrangements underwent lobectomy (LT),

193 independently of US nodule features, in line with the previously demonstrated increased cancer

194 risk associated with these mutations (18). Patients with class III lesions without a genetic variation

195 in the studied genes underwent a second FNAB and then underwent lobectomy if the cytological

196 diagnosis was confirmed; otherwise the patients were managed according to the new BSRTC

197 system. Finally, patients with class II lesions underwent clinical follow-up.

198

199 <u>Cytology, molecular testing, US and pathology findings</u>

200 Cytological results and genetic alteration frequencies are displayed according to BSRTC classes in

201 Table 1. Among 940 FNAB, 134 displayed at least one mutation (14.2%), specifically a

202 *BRAFV600E* mutation in 4.2% of all nodules, *RAS* mutations in 3.4% (25 at *N-RAS* codon 61, 1 at

203 *H-RAS* codon 13, 1 at *H-RAS* codon 61, 2 at *K-RAS* codon 12, 1 at *K-RAS* codon 13, 2 at *K-RAS*

204 codon 61), and *RET/PTC* rearrangements in 7.3% (3.9 % *RET/PTC1* and 3.4 % *RET/PTC3*). The

205 highest incidence of *RAS* mutations was found within BSRTC class III and class VI samples,

206 while the highest incidence of *RET/PTC* rearrangements was found among BSRTC class I

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207   samples (of which about 30% was operated on and had a benign histology) and among BSRTC

208   class III and VI samples (Table 1).

209   The presence of a *BRAFV600E* mutation was significantly associated (p<0.01) with

210   hypoechogenicity, microcalcifications and a diameter <1 cm. *RAS* mutations were significantly

211   (p<0.01) associated with isoechogenicity and a diameter >1 cm. *RET/PTC3* rearrangements were

212   significantly (p<0.01) associated with isoechogenicity on US.

213   Overall, 72 patients underwent TT and 45 patients underwent LT, which was completed in 5

214   patients (11.1% of LT), for a total of 117 operated patients**.** Among these, 62 patients (52.1%) had

215   an indeterminate lesion on cytology: 23 AUS/FLUS (class III), 17 FN (class IV) and 22 SM (class

216   V). The presence of a cancer was histologically confirmed in 72 patients (61.5% of operated

217   patients), including 70 papillary thyroid cancers (PTC; 96.05%), 1 follicular thyroid cancers

218   (FTC) and 1 anaplastic thyroid cancer (ATC). Among the patients with a final malignant

219   histology, more than half carried one or more somatic genetic alteration and displayed stage I

220   disease (Table 2).

221   In particular, 40 patients who displayed a somatic *BRAF V600E* mutation (including 6 who also

222   displayed a *RET/PTC* rearrangement) underwent TT and had a PTC on final histology.

223   Among the 31 patients who displayed an isolated somatic *RAS* mutation, 10 were submitted to LT

224   and 1 to TT. Histology revealed the presence of a cancer in 2 cases, including 1 ATC and 1 FTC

225   (the latter initially submitted to LT and then to completion thyroidectomy). The remaining 9

226   patients that were operated on showed a follicular adenoma (FA) in 6 cases and hyperplastic

227   nodules (HN) in 3 cases. Moreover, one patient with a malignant cytology, displaying a somatic

228   *RAS* mutation, was not operated on due to several co-morbidities. The remaining 19 patients

229   refused surgery, mostly because of the finding of a benign cytology.

230   The presence of a *RET/PTC* rearrangement was found in 69 FNAB, 6 of which also harbored a

231   *BRAFV600E* mutation and were therefore submitted to TT; one patient carried also a *RAS*

232   mutation and was submitted to LT with final histology of a FA; one was to have both *RET/PTC*

9

233 rearrangements and was submitted to TT with a final histology of PTC. Among the 62 patients

234 displaying an isolated *RET/PTC* rearrangement, 5 underwent TT (in the presence of a BSTRC

235 class V in 2 patients and class VI in 3 patients) and 19 underwent LT. Histology revealed the

236 presence of a cancer in 5 cases (all PTC), while 11 lesions were FA and 8 HN. The remaining 38

237 patients refused surgery, mostly because of the finding of a benign cytology. No correlation was

238 found between the presence of a malignant lesion and the amount of *RET/PTC* rearranged mRNA,

239 preventing the identification of a threshold value that discriminates benign from malignant lesions.

240

241 Indeterminate lesions

242 We then evaluated cytology, molecular testing and pathology findings in the group of

243 indeterminate nodules, which were included in the whole group described above.

244 We found that 37 (26.4%) of the 140 cytologically indeterminate lesions (corresponding to 14.8 %

245 of all FNAB), including 19 class III, 7 class IV and 11 class V lesions, displayed at least one

246 genetic alteration. Among these patients, 2 refused LT (class III cytology) and 35 underwent TT.

247 Final histology showed 24 thyroid cancers (23 PTC and 1 FTC), 8 FA and 3 HN. Among the 23

248 identified PTCs, 21 carried a somatic *BRAF V600E* mutation.

249 Among the 103 patients with a cytologically indeterminate lesion not displaying a genetic

250 alteration, all the 11 patients with a class V lesion underwent TT, with a final histology of 10 PTC

251 and 1 HN. Ten out of 30 patients with class IV lesions accepted to undergo LT, with a final

252 histology of 3 PTC (then submitted to completion thyroidectomy) and 7 FA. All 62 patients with a

253 class III lesion underwent a second FNAB that confirmed an indeterminate lesion in 33 cases; 6 of

254 these patients accepted to undergo LT, and the final histology showed 1 FTC, 4 FA and 1 HN.

255 Cytology showed a benign lesion in the other 29 patients who were then re-classified as BSRTC

256 class II and subsequently followed with US. The management of these patients was chosen

257 according to the ATA guidelines (8), in order to avoid unnecessary surgery in keeping with the

258   low cancer risk of BSRTC class III nodules (in contrast with the higher cancer risk of BSRTC

259   class IV and V nodules).

260   Taken together, in our series malignancy rates in each BSRTC class overlap those described by

261   Cibas et al. (14). The cancer risk in thyroid nodules with indeterminate cytology according to

262   BSRTC classification and genetic alterations is shown in Table 3.

263

264   Diagnostic value of cytology and molecular analyses

265   The diagnostic value of cytology and of the studied mutational analyses is reported in Table 4a,

266   which also reports the results obtained by performing the three available genetic analyses in

267   combination. Our data show that cytology displays optimal PPV and specificity, while sensitivity

268   for thyroid cancer is low. When performed alone, *BRAFV600E* analysis shows, as compared to

269   cytology, a significantly higher diagnostic sensitivity ($p<0.05$), which increases by 20.8%

270   ($p<0.01$) when the two evaluations are performed together (Table 4b). On the other hand, the

271   presence of *RAS* mutations and *RET/PTC* rearrangements shows a very low sensitivity for thyroid

272   cancer when evaluated alone (Table 4a) and does not significantly increase the diagnostic

273   sensitivity of cytology (Table 4b). In addition, the increased sensitivity recorded when all three

274   genetic analyses are performed in combination is not significantly higher as compared to the

275   sensitivity obtained by performing *BRAFV600E* analysis alone, even when combined with

276   cytology. These data indicate that, in our setting, *BRAFV600E* analysis suffices to increase the

277   diagnostic sensitivity of cytology for thyroid cancer.

278   We then evaluated the diagnostic sensitivity of the genetic analysis panel in the subset of the

279   indeterminate lesions, in order to understand whether and which mutation testing might be helpful

280   in this group. We found that the diagnostic sensitivity for thyroid cancer of the three genetic

281   analyses in the indeterminate group, performed alone or in combination, overlaps that identified in

282   the whole group. We then analyzed each BSRTC class included in the indeterminate group (Table

283   4c) and found that the diagnostic sensitivity for thyroid cancer reaches 90% in class III when

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284   *BRAFV600E* analysis is performed. This value does not change when *RAS* mutations and

285   *RET/PTC* rearrangements are simultaneously included. In class IV and V samples, when all three

286   genetic abnormalities are analysed in combination, the diagnostic sensitivity for cancer is greater

287   as compared to *BRAFV600E* alone, but the difference is not statistically significant. In addition,

288   the analysis of *RAS* mutations and *RET/PTC* rearrangements does not seem to be important to

289   further increase the high NPV of *BRAFV600E* analysis in class III and IV samples.

290

291   **DISCUSSION**

292   This prospective study confirms the diagnostic utility of assessing the presence of a *BRAFV600E*

293   mutation (16). On the other hand, the investigation of two additional genetic abnormalities (*RAS*

294   mutations and *RET/PTC* rearrangements) did not significantly increase the diagnostic sensitivity

295   of cytology towards thyroid cancer in this cohort, even in the category with indeterminate lesions.

296   Despite the fact that the techniques employed in our study are very similar to those employed by

297   others (5, 15, 18), the results do not overlap. It should be noted that the method employed here to

298   assess *RET/PTC* rearrangements displayed a 10-fold higher sensitivity as compared to that

299   employed by Nikiforov et al. (15, 18), but provided low sensitivity and specificity in detecting

300   malignant lesions. Therefore, the identification of *RET/PTC* rearrangements by a very sensitive

301   method may not be useful to increase FNAB diagnostic sensitivity for thyroid cancer. These data

302   suggest that the contribution of this genetic marker to pre-surgical diagnosis of thyroid nodules

303   may not be so relevant, since we found a very high incidence of *RET/PTC* rearrangements also in

304   benign lesions.

305   US characteristics provide the basis to perform FNAB (8) and often accurately predict the

306   presence of a *BRAFV600E* mutation (20). In our hands, the presence of a *BRAFV600E* mutation

307   was significantly associated with hypoechogenicity, microcalcifications and a diameter <1 cm,

308   strengthening the evidence that nodules displaying these US characteristics very likely reflect the

309   presence of a cancer. Our study highlights, for the first time, that *RAS* mutations and *RET/PTC*

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310 rearrangements correlate with specific US findings (i.e. isoechogenicity and diameter >1 cm).

311 However, these genetic abnormalities do not indicate the presence of a cancer with high accuracy

312 in our population, and therefore the related US characteristics cannot be taken into account as

313 predictive of cancer.

314 The distribution of our samples among BSRTC classes is in line with literature data, indicating

315 that the investigated nodules had been selected according to the indications of the ATA guidelines

316 (8). In particular, more than 80% of FNAB cytologies turned out to be a benign lesion and ~12%

317 of the samples displayed an indeterminate cytology. The latter result is very similar to the

318 percentage of indeterminate lesions that were retrieved in our previous study (17) which included

319 an unselected nodule population, indicating that the application of strict selection criteria for

320 FNAB does not influence the number of indeterminate lesions. While the percentage of malignant

321 lesions identified by cytology in our series (2.9%) is comparable to the literature data, the

322 incidence of ND reports is quite high (3.5%). This may be due to the fact that the retrieved FNAB

323 material was used for several diagnostic procedures, which may have reduced the sample quantity

324 dedicated to cytology.

325 The present series shows that 14.2% of the investigated nodules harbored at least one mutation, a

326 higher incidence than the previously reported ~9% (18), probably due to the different inclusion

327 criteria. In addition, 6% of mutated FNAB samples displayed more than one genetic alteration,

328 confirming that *BRAF* and *RAS* mutations, as well as *RET/PTC* rearrangements, are not mutually

329 exclusive, as previously indicated (21). Our data also show that the applied FNAB criteria allowed

330 diagnosing thyroid cancers at an early stage of disease, since 65.3% of the diagnosed cancers were

331 Stage I. In addition, nearly 50% of Stage I cancers had a negative cytology but displayed at least

332 one genetic alteration, most commonly a *BRAFV600E* mutation, which allowed to establish a

333 correct diagnosis. These data indicate that *BRAFV600E* mutation analysis helps in identifying

334 PTC at an earlier stage, possibly resulting in a more conservative treatment with potential

335 consequences on patient health and healthcare resources. Moreover, 76% of Stage III and IV

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336   cancers displayed a genetic alteration, in line with the hypothesis that the latter may characterize a

337   more aggressive behavior (22, 23), as previously indicated (24). Last, but not least, the applied

338   protocol allowed to correctly diagnose 31 out of 46 false negative lesions on cytology as cancers,

339   corresponding to 43% of the diagnosed malignant lesions. Among these 31 patients, 21 harbored a

340   *BRAFV600E* mutation and an indeterminate cytology, and were therefore submitted to TT rather

341   than to a diagnostic LT. Moreover, 7 patients were submitted to TT only on the basis of positivity

342   for a *BRAFV600E* mutation and turned out to have a PTC (6 Stage I and 1 Stage III). The latter

343   finding strengthens the evidence that *BRAFV600E* mutation analysis facilitates early diagnosis. On

344   the other hand, in our settings *RAS* mutations have a poor diagnostic value, in keeping with their

345   rarity, and are predominantly associated with follicular lesions, mainly represented by FA that

346   may, in part, be considered as precursors of malignant lesions (25). In keeping with the latter

347   hypothesis, *RAS* mutated cancers were characterized by an aggressive histology and a high disease

348   stage. In our patients, each *RET/PTC* rearrangement was nearly as frequent as *BRAFV600E*

349   mutations, but had a poor diagnostic value since the rearranged lesions were mostly found in

350   benign nodules (64.5% of the cases), contrary to what observed by Cantara et al. (5) and Nikiforov

351   et al. (18), but in line with Marotta et al. (26), even if a prognostic significance cannot be ruled out

352   (27). These differences may be due to different genetic backgrounds and to geographic factors, but

353   may also be due to the applied selection criteria. Among the samples harboring *RET/PTC*

354   rearrangements, the 11 PTC cases had a *BRAFV600E* mutation and/or a suspicious or malignant

355   cytology, and were therefore submitted to TT independently of the presence of a *RET/PTC*

356   rearrangement.

357   A previous report (18) showed an increased diagnostic sensitivity for thyroid cancer in a large

358   group of indeterminate nodules submitted to multiple genetic analyses (including *BRAFV600E*

359   and *RAS* mutations as well as *RET/PTC1*, *RET/PTC3* and *PAX8/PPARγ* rearrangements). The

360   study showed a high NPV for this panel of molecular markers, indicating that the absence of a

361   genetic mutation very likely excludes the presence of a malignant lesion. On the contrary, we did

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362 not obtain high NPV values in the indeterminate group when performing the three analyses

363 together (*BRAFV600E* and *RAS* mutations, as well as *RET/PTC1* and *RET/PTC3* rearrangements),

364 but we found a high NPV for *BRAFV600E* mutation analysis alone, which is even higher in class

365 III nodules. The latter finding, together with the low cancer risk, suggests that in the absence of a

366 *BRAFV600E* mutation, diagnostic LT may not be necessary in class III nodules. In class IV

367 nodules without mutations, we found a slightly higher cancer risk, which importantly increased

368 when a *RAS* mutation was present. These data, together with a suboptimal NPV of *BRAFV600E*

369 analysis in class IV lesions, do not support a conservative management in these settings (i.e.

370 avoiding a LT). On the other hand, cancer risk is high in class V nodules, indicating that an

371 aggressive surgical management (i.e. TT) is justified in these patients, independently of the

372 presence of a mutation, like in class VI lesions. Taken together, these data demonstrate that,

373 among the investigated molecular markers, only *BRAFV600E* mutation may modify patient

374 management and has an impact on the surgical approach. Therefore, our data concerning

375 indeterminate lesions are only partially in keeping with previous findings (18), probably due to the

376 different inclusion criteria, that may play an important role in molecular studies.

377 In conclusion, our results confirm that *BRAFV600E* analysis performed in all BSRTC classes

378 increases the diagnostic sensitivity of cytology for thyroid cancer, which is not further enhanced

379 by investigating the presence of *RAS* mutations or *RET/PTC* rearrangements, even among

380 indeterminate nodules. In addition, our data demonstrate that *BRAFV600E* analysis, when

381 negative, may be useful to identify class III nodules at very low risk of being cancerous,

382 suggesting that these cases may be treated more conservatively and do not need to be submitted to

383 a LT. Moreover, we conclude that *BRAFV600E* analysis is useful to diagnose thyroid cancer at an

384 early stage, possibly reducing the clinical impact of a delayed diagnosis, which also implicates

385 higher costs for the patients and for the healthcare system.

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388 **ACKNOWLEDGEMENTS**

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394 **DISCLOSURES**

395 EdU received consulting fees from Novartis and Pfizer. MCZ received consulting fees from

396 Novartis and Genzyme. The other Authors have nothing to disclose and have no conflict of

397 interest.

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Table 1: Genetic alterations and their frequencies in each BSTRC class nodules

Genetic alteration (n)	BSTRC classes						
	I	**II**	**III**	**IV**	**V**	**VI**	**Total**
BRAF V600E	4	3	7	4	6	10	**34**
BRAF and *RET/PTC 1*	0	0	2	0	1	1	**4**
BRAF and *RET/PTC 3*	0	0	0	0	1	1	**2**
RAS	1	21	4	2	1	2	**31**
RAS and *RET/PTC 3*	0	1	0	0	0	0	**1**
RET/PTC-1	2	25	3	0	1	1	**32**
RET/PTC-3	4	19	3	1	1	1	**29**
RET/PTC-1 and *-3*	0	0	0	0	0	1	**1**
Total samples with genetic alteration(s)	11	69	19	7	11	17	**134**
None	22	699	33	30	11	11	**806**
All samples	33	768	52	37	22	28	**940**
Genetic alteration frequence (%)							
BRAF V600E	12.1	0. 4	17.3	10.8	36.3	42.8	**4.2**
RAS	3	2.8	7.7	5.4	4.5	7.1	**3.4**
RET/PTC-1 and *RET/PTC-3*	18.2	5.8	15.4	2.7	18.2	17.8	**7.3**
Total (s)	**33.3**	**9**	**36.5**	**18.9**	**50.0**	**60.7**	**14.2**

Table 2: Distribution according to TNM stages and the presence/absence of a genetic alteration.

TNM staging (AJCC/UICC)	Thyroid cancers		
	Genetic alteration		Total
	positive	negative	
I	28	19	47
II	0	0	0
III	13	6	19
IV	6	0	6
Total	**47**	**25**	**72**

Table 3: Cancer risk in thyroid nodules with indeterminate cytology according to BSTRC
classification and genetic alteration

%	class III	class IV	class V	Indeterminate cytology
Cytology alone	19.2	21,6	90,9	27,1
Any mutation	47,3	71,4	90,9	63,1
BRAF	100	100	100	100
RAS	0	50	0	14,2
RET/PTC-1	40	-	100*	57,1
RET/PTC-3	0	0	100*	33,3
No mutations	3	10	90,9	13,5

*The patients with a PTC displaying *RET/PTC* rearrangements also had a *BRAFV600E* mutation or
a class V or a class VI BSTRC cytology.

Table 4a: Diagnostic value of cytology and of genetic analyses in all 940 samples

	Cytology	*BRAF*	*RAS*	*RET/PTC*	All genetic analyses
PPV	100	100	25	34,4	63,3
NPV	50	58,4	34,3	28,2	34,2
sensitivity	37,5	55,6	4,2	15,3	66,7
specificity	100	100, 0	80	53,3	31
accuracy	61,5	72,6	33,3	29,9	53,8

Table 4b: Diagnostic value of cytology combined with genetic analyses in all 940 samples

	Cytology combined with			
	BRAF	*RAS*	*RET/PTC*	All genetic analyses
PPV	100	76,3	61,1	66,7
NPV	72,6	45,6	38,1	51,9
sensitivity	76,4	40,3	45,8	82,2
specificity	100	80	53,3	31,8
accuracy	85,5	55,6	48,7	63,2

Table 4c: Diagnostic value of genetic analyses in the 140 indeterminate lesions according to BSRTC classification

	Class III		Class IV		Class V	
	BRAF	all genetic analyses	*BRAF*	all genetic analyses	*BRAF*	all genetic analyses
PPV	100	52,9	100	71,4	100	90,9
NPV	92,9	83,3	69,2	70	14,3	9,1
Sensitività	90	90	50	62,5	40	50
Specificità	100	38,5	100	77,8	100	50
Accuracy	95,7	60,9	76,5	70,6	45,5	50