

CLINICAL STUDY

Diagnostic and prognostic value of immunocytochemistry and *BRAF* mutation analysis on liquid-based biopsies of thyroid neoplasms suspicious for carcinoma

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Abstract

Objective: In the field of fine-needle aspiration cytology, the category of suspicious for malignancy (SM) thyroid lesions, that bears 55–85% risk of malignant histology, is a challenging topic in which morphology alone is not always able to make a correct diagnosis. Recently, immunocytochemistry (ICC) has been referred to as helpful in differentiating low- and high-malignant risk lesions and *BRAF* activating mutations have been identified in a significant amount of papillary thyroid carcinomas (PTC). The introduction of the liquid-based cytology (LBC) may simplify the application of these techniques to thyroid cytology.

Design: Our aim is to evaluate the diagnostic and prognostic role of both ICC and *BRAF* mutation for the SM category on LBC.

Methods: From October 2010 through June 2011, 113 LBC cytological cases (including 37 SM and 76 PTC) underwent surgery. All cases were studied for *BRAF* mutation and ICC.

Results: ICC resulted positive in 26 (86.6%) histologically malignant SM with 15 of which (40.5%) expressing a *BRAF* mutation. Overall, 63 cases showed a *BRAF* mutation resulting in PTC. Concerning the prognostic role of *BRAF* mutation for the two categories, we reported a significant correlation with multifocality, nodal involvement and extra-capsular invasion ($P < 0.0001$).

Conclusions: Special techniques such as ICC and molecular markers might be successfully carried out on LBC-processed material. For both categories, ICC is more sensitive whereas *BRAF* analysis is an interesting support due to its high specificity adding a prognostic value in both SM and PTCs.

European Journal of Endocrinology 168 853–859

Introduction

Thyroid nodules are a common finding in the general population, taking into account both non-neoplastic and neoplastic entities, representing an area fraught with controversy for which fine-needle aspiration cytology (FNAC) is the most important and generally the first diagnostic tool (1, 2, 3).

FNAC is a worldwide application because of its simplicity, safety and cost-effectiveness, leading to a correct diagnosis in more than 70% and to a correct clinical approach in more than 90% of patients (1).

Nevertheless, the majority of all thyroid FNACs are benign, with 5–10% being 'malignant' and the remaining 20% representing a field in which different entities are included. This latter group has been discussed by different international classifications, such as the Bethesda System of Reporting Thyroid

Cytopathology, in which three subcategories have been defined bearing different risks of malignancy and different treatments. On the other hand, the Italian and British cytological classifications encompass only two categories, follicular neoplasm (FN) and suspicious for malignancy (SM), the former including the majority of FN from the Bethesda classification (4).

Apart from these morphological problems, mainly ascribed to the number of false-positive cases, the current recommendation for SM management is reflected in a large consensus for a lobectomy, although in some institutions, a total thyroidectomy is the first initial surgical choice. Looking for an answer, a growing number of experts in the field have increasingly found specific markers (both immunocytochemical and molecular markers) on cytological samples.

The expression of immunohistochemical markers has been proposed as an additional tool for discriminating

malignant tumours at histology regardless of the presence of capsular and/or vascular invasion. Hector Battifora mesothelial-1 (HBME-1) and Galectin-3 have reached the highest specificity and sensitivity in malignant lesions even if none of the antibodies studied so far have shown a diagnostic accuracy sufficient for its use as single marker of malignancy (2, 3, 5, 6, 7, 8).

Furthermore, some molecular pathway alterations play a pivotal role in thyroid cancer and, importantly, represent a precocious step in the tumorigenic process justifying their use as uncountable markers of malignancy. The *V600E BRAF* mutation is typically found in 29–69% of classical papillary thyroid carcinomas (PTC), the most common thyroid malignancy, and in 80% of the tall cell variant (TCV), while it is less commonly identified in the follicular variant of papillary carcinoma (FVPC) (9, 10, 11, 12, 13). The evidence that *BRAF* mutation could have a diagnostic and prognostic role through progression in a less favourable prognosis has assumed a critical role in the field of cytological thyroid follicular lesions (10, 11, 12, 14).

The application of these special techniques (e.g. molecular markers and immunocytochemistry (ICC)) may show some hitches when applied to conventional cytology. For this reason, in 1996, liquid-based cytology (LBC) has gained popularity as an alternative technique for collection and preparation of cytological specimens based on a methanol-preserved solution and processed with a semi-automated device (1, 2, 3, 15, 16, 17, 18).

It is well-known that there are conflicting opinions and controversial data regarding the efficacy of LBC, although several positive aspects in term of cost-effectiveness, time-sparing, simple application of ancillary techniques such as ICC and molecular biology up to 3–4 months on LBC stored material must be underlined (3, 4, 19, 20, 21, 22, 23).

The aims of this study are i) to evaluate the efficacy and feasibility of LBC for the application of ICC and *BRAF* molecular analysis to thyroid aspiration cytology and ii) to turn out the diagnostic and prognostic role of these techniques on both SM and malignant categories.

Materials and methods

From January 2011 through December 2011, a total number of 113 prospective cases (including 37 SM and 76 positive for malignancy) out of 2721 total thyroid FNAB recorded in the files of the Division of Anatomic Pathology and Histology of the Catholic University, 'Agostino Gemelli' Hospital of Rome (Italy), underwent surgery. All 113 cases underwent *BRAF* analysis and were studied with an immunopanel comprising HBME-1 and Galectin-3. The cases in which the immunopanel results were discordant were further studied for the expression of cytokeratin 19 (CK19). A panel of three antibodies would have been a preferable option but obtaining routinely those

additional slides and storing material for molecular analysis in a thyroid FNA often is not possible. All the nodules were evaluated under sonographic guidance (US), mostly by surgeons and endocrinologists, and processed with the LBC method Thin Prep 2000 (Hologic Co., Marlborough, MA, USA). The series included 50 male and 63 female patients with a median age of 42 years (range 20–70 years). The high M/F rate can probably be ascribed to some bias of our surgical series. All aspirations (usually two passes for each lesion) were performed with 25–27 G needles; no fast assessment of adequacy of the material was done. The nodule sizes ranged from 0.4 to 7 cm. All the sub-centimetre lesions were discovered during routine US thyroid check-up performed in the Centre for Thyroid Diseases of the Departments of Endocrinology and Endocrine Surgery of our hospital. All patients had been appropriately informed regarding use of the LBC method for processing their aspiration samples and a written informed consent was signed by all of them.

The aspirated material was fixed with the haemolytic and preservative solution Cytolit after rinsing the needle in this solution. The cells were spun at 50 *g* and then the sediment was transferred into the Preservcyt solution to be processed with the T2000 automated processor according to the manufacturer's suggestions. The resulting slide was fixed in 95% ethanol and stained with Papanicolaou while the remaining material was stored in the Preservcyt solution at room temperature for 3–4 months to be used for eventual additional investigations. Both ancillary techniques (ICC and *BRAF* analysis) were applied simultaneously to the cytological diagnosis. These techniques can be performed also when the remaining material is at about 2 ml eluted in 5 ml Preservcyt solution.

The lower limit for the adequacy for each sample was established, according to the British RCPATH classification, in six groups of thyroid follicular epithelial cells within the submitted slides, each of them with at least ten well-visualised epithelial cells (24).

The cytological cases were classified according to the Italian Working Group SIAPEC-IAP classification, which shows several overlapping features with both the Bethesda and the British RCPATH classifications, especially for the SM category (4, 24, 25).

The above-mentioned categories are defined as follows: TIR1, inadequate or hemorrhagic; TIR2, non-neoplastic lesion; TIR3, follicular lesion/suspected FN; TIR4, SM and TIR5, positive for malignant neoplasm. Our cytological series presented the following distribution of diagnoses for the reference year: 6.5% TIR1 (non-diagnostic), 79% TIR2 (non-neoplastic), 10.3% TIR3 (indeterminate), 1.7% TIR4 (suspicious) and 2.5% TIR5 (malignant).

The morphological diagnosis of TIR4/SM was achieved in the presence of follicular thyroid cells with nuclear pleomorphisms, irregularities and grooves but without any nuclear inclusion.

Immunocytochemical analysis

Immunocytochemical stainings were carried out with the avidin–biotin peroxidase complex on LBC slides using the following antibodies: HBME-1 (Dako, Glostrup, Denmark, 1:100 dilution) and Galectin-3 (Ventana, Tucson, AZ, USA, 1:100 dilution) and CK19 (Dako, 1:100 dilution). The slides were washed three times in PBS and then pre-incubated in normal veal serum with PBS (1:50) for 20 min before overnight incubation at 4 °C with the primary antibody. Then, the slides were washed three times with PBS and incubated with the biotinylated secondary antibody conjugated with the avidin–biotin–peroxidase complex (Ventana). The reaction was developed using 3,3'-diamino-benzidine as a chromogen. All slides were counterstained with haematoxylin for 5 s, rinsed in water three times and then mounted for the microscopic examination. The positivity was assessed, for each cytological case, when at least 50% of cells showed a strong cytoplasmic positivity. This arbitrary 50% ICC cut-off was established based on the histological diagnoses. The positivity of each case was defined only when a concomitant positive expression of the two immunomarkers was detected. Positive controls were represented by typical papillary carcinomas and negative controls by histiocytes and lymphocytes identified in most of the thyroid slides.

Histology

All surgical specimens were fixed in 10% buffered formaldehyde, embedded in paraffin, and the 5 µ-thick microtomic sections were stained with haematoxylin–eosin. The BRAF mutational analysis was also performed on DNA extracted by surgical specimens, containing at least 70% of tumour. The concordance of immunohistochemistry and mutational analysis between the surgical and LBC samples was 100%. All the fibro-adipose tissue close to the thyroid gland was included for the lymph node research.

BRAF mutational analysis

DNA extraction was performed on fine-needle cytological sample ThinPrep 2000 (Hologic Co.) and paraffin block using the QIAamp tissue kit (Qiagen). The percentage of disease-specific cells for molecular analysis was at least 50% in all LBC samples and 70% in surgical specimens. We assessed the quantity and quality of the DNA spectrophotometrically (E260, E260:E280 ratio, spectrum 220–320 nm; Biochrom, Cambridge, UK) and by separation on an Agilent 2100 Bioanalyzer (Palo Alto, CA, USA).

BRAF genes (exons 11 and 15) were amplified using the following primers: for exon 11, forward 5'-TTA TTG ATG CGA ACA GTG AAT AT-3' and reverse 5'-TTA CAG TGG GAC AAA GAA TTG-3'; for exon 15, forward 5'-TCA TAA TGC TTG CTC TGA TAG GA-3' and reverse

5'-GGC CAA AAA TTT AAT CAG TGG A-3'. Briefly, DNA (100–200 ng) was amplified in a mixture containing 1×PCR buffer (20 mM Tris (pH 8.3), 50 mM KCl and 1.5 mM MgCl₂), dNTPs (200 mM each), primers (20 pM each) and 0.5 U of GoTaq polymerase (Promega) in a final volume of 25 µl. PCR conditions were as follows: initial denaturation at 95 °C for 8 min followed by 35 cycles at 95 °C for 40 s, 55 °C for 40 s and 72 °C for 40 s. After visualisation onto agarose gel, PCR products were treated with ExoSAP-IT (USB Corp., Cleveland, OH, USA) following the manufacturer's protocol, amplified with BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) using forward and reverse primers and sequenced with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Water was used as a negative control.

Statistical analysis

Statistical analysis was performed using a commercially available statistical software package (SPSS 10.0) for Windows (Microsoft). Comparison of categorical variables was performed by χ^2 statistic using the Fisher's exact test when appropriate. A *P* value <0.05 was considered significant.

Results

One hundred and thirteen FNAC cases processed by LBC underwent surgery. The series includes 37 SM and 76 positive for malignancy/TIR5. All the SMs and malignancies were studied with the application of an immunopanel made up of HBME-1 and Galectin-3. All 113 cases were also evaluated for BRAF mutation. The 76 positive for malignancy/TIR5 were histologically confirmed while 30 out of 37 SM (81%) were found to be malignant. Table 1 shows the distribution of cytological diagnoses with ICC analysis and BRAF mutation. The ICC analysis, performed in the SM group, was considered informative only in the cases with a concordant positive or negative immunopanel based on the evidence that single immune-marker positivity is not diagnostic alone (2, 5). The immunopanel application resulted in 11/37 (30%) negative SMs and 26/37 (70%) positive SM cases, with four malignant cases showing positivity only for HBME-1 (Table 1).

Table 1 Cytohistological comparison between BRAF and ICC results.

	FNAC	Malignant histology	BRAF+	ICC–	ICC+
SM (TIR4)	37	30	15	11	26
PTC (TIR5)	76	76	48	0	76
Overall	113	106	63	11	26

SM, suspicious for malignancy (TIR4, TIR5: positive for malignancy); ICC, immunocytochemistry.

Table 2 Comparison between ICC and *BRAF* analysis with the histological diagnosis of SM.

	ICC−, BRAF−	ICC+, BRAF+	ICC−, BRAF+	ICC+, BRAF−
BL	7	0	0	0
PC	1 ^a	12	0	5
FVPC	3 ^a	1	0	6
TCV	0	2	0	0

BL, benign lesion; PC, papillary carcinoma (classic variant); FVPC, follicular variant of PC; TCV, tall cell variant of PC; ICC, immunocytochemistry; SM, suspicious for malignancy (TIR4, TIR5: positive for malignancy).

^aAll these cases showed a discordant panel (two FVPC positive for HBME-1 and cytokeratin 19 whereas one PC and one FVPC only for HBME-1).

In these four discordant cases, we also evaluated that CK19 was positive in two out of the four (50%). All our papillary carcinomas resulted positive for both immunomarkers and were used as positive control cases. The correlation between histological type and *BRAF* detection highlighted mutated *BRAF* in 63 cases out of 113 (48 TIR5 and 15 SM), while 50 out of 113 (28 TIR5 and 22 SM) showed a wild-type sequence. All cases except one (with a double mutation involving two nucleotides at codon 600, GTG>GAA) had a point mutation of GTG to GAG at codon 600 of *BRAF* exon 15. We did not find any mutation of *BRAF* exon 11 (Table 1). The sensitivity and specificity of this mutation assay in our laboratory are 85 and 97% respectively (26). The concordance of mutational analysis between the surgical and LBC samples was 100%.

In Table 2, we compared the results of ICC and *BRAF* analysis with the histological diagnosis of the SM category, arranging the results in four sub-groups. In detail, the 11 SM with negative immunopanel and *BRAF* analysis included the seven benign histology and four thyroid cancers (one PTC and three FVPC). All the remaining 26 SMs, positive for the immunopanel but with 15 out of 26 *BRAF*-positive detections, were histologically malignant (17 PTC, two TCV and seven FVPC; Table 2).

The data concerning *BRAF* indicated that all the seven benign lesions were *BRAF* wild-type and 15 of the 30 malignant cases expressed *BRAF* mutation. These cases were stratified as 12 classical variants of PTC, two TCV and one in the FVPC subgroup. This result confirms the idea of a more diagnostic role of a positive concordant ICC ($P=0.0007$, OR 55) for the cytological diagnosis of the FVPC histotype (seven cases identified by ICC and just one by *BRAF* mutation).

In Table 3, we analysed the distribution of histological diagnosis and of parameters of aggressiveness (e.g. multifocality, extra-capsular thyroid invasion and lymph-node metastases) based on the cytological classification. The final histological diagnosis resulted in seven benign follicular adenomas (not specified in that table) and 106 malignant diagnoses including 79 PC (61 in the positive for malignancy/TIR5 group and 18 in SM), 20 FVPC (ten in positive for

malignancy/TIR5 and ten in SM) and seven TCV (five in positive for malignancy/TIR5 and two in TCV). No false-positive case was found in the positive for malignancy/TIR5 category. We also analysed the data concerning the three parameters of aggressiveness expressed by 47 extra-capsular thyroid invasion (33 positive for malignancy/TIR5 and 14 SM), 41 multifocal cancers (24 positive for malignancy/TIR5 and 17 SM) and 42 lymph node metastatic disease (27 positive for malignancy/TIR5 and 15 SM). All TCV cases were multifocal, with extra-capsular invasion and lymph node involvement (Table 3).

In Table 4, we analysed the relationship between *BRAF* mutations and parameters of aggressiveness for the two analysed categories of SM and positive for malignancy/TIR5.

The presence of a *BRAF* mutation, for the SM category (Table 4), was significantly linked with evidence of multifocality ($P<0.0001$, OR 0.004, 95% CI 0.0002–0.0880), with extra-thyroidal extension ($P<0.0001$, OR 0.025, 95% CI 0.0037–0.171) and nodal metastases ($P<0.0001$, OR 0.015, 95% CI 0.0019–0.1233).

Concordantly, also in the positive for malignancy/TIR5 group (Table 4), the cases with *BRAF* expression showed a higher bilateral localisation of thyroid cancer ($P=0.0003$, OR 11.00, 95% CI 2.34–51.65), more frequent lymph node metastases ($P<0.0001$, OR 14.13, 95% CI 3.01–66.3) and extra-capsular infiltration ($P=0.03$, OR 3.26, 95% CI 1.17–9.1).

In the SM entity, the sensitivity, specificity, diagnostic accuracy and negative and positive predictive values of immunostainings were 86.6, 100, 89, 63 and 100% respectively. The same evaluation about *BRAF* analysis in the same group revealed 50% sensitivity, 100% specificity, 60% diagnostic accuracy and 32% negative and 100% positive predictive values respectively.

Discussion

Although thyroid FNAC represents the most reliable diagnostic approach for establishing the correct diagnosis of either benign or malignant thyroid nodules, a percentage spanning from 10 to 30% of all cytological samples is included in categories characterised by very

Table 3 Distribution of malignant histological diagnosis and of parameters of aggressiveness based on the cytological classification.

	PC	FVPC	TCV	EC invasion	Multi- focality	LN mets
SM (TIR4)	18	10	2	14	17	15
PTC (TIR5)	61	10	5	33	24	27
Overall	79	20	7	47	41	42

PC, papillary carcinoma (classic variant); FVPC, follicular variant of PC; TCV, tall cell variant of PC; ICC, immunocytochemistry; SM, suspicious for malignancy (TIR4, TIR5: positive for malignancy, EC, extra-capsular; LN, lymph node; mets, metastases).

Table 4 Distribution of multifocality, capsular involvement and nodal metastases based on *BRAF* molecular pattern in the SM and TIR5 lesions.

	<i>BRAF</i> +	<i>BRAF</i> -	<i>P</i>
SM lesion			
Unifocal tumour	0	20	<0.0001
Multifocal tumour	15	2	
Intrathyroid tumour	3	20	<0.0001
Extra-thyroid tumour	12	2	
Node negative	2	20	<0.0001
Node positive	13	2	
TIR5 lesion			
Unifocal tumour	26	26	0.0003
Multifocal tumour	22	2	
Intrathyroid tumour	23	20	0.03
Extra-thyroid tumour	25	8	
Node negative	23	26	<0.0001
Node positive	25	2	

different cancer risk (spanning from 5–15 to 60–75%) and variable clinical actions (4, 27, 28, 29).

Even though SM morphology alone may be sufficient for the detection of atypia and nuclear pleomorphisms especially in expert cytological hands, several papers (including a recent one by Mahajan *et al.* (28)) highlighted the pitfalls of the morphology alone, which can lead to inappropriate treatment (lobectomy vs total thyroidectomy or frozen section), additional morbidity and higher health care costs.

A recent review article by Correia-Rodrigues *et al.* (30) and other papers have highlighted the increasing application of ancillary techniques to thyroid cytology focusing on immunocytochemical panels as a good choice to discriminate between low- and high-malignant risk lesions that result in an overall diagnostic accuracy spanning from 81 through 92% with the concordant positive panel (3, 5, 29, 30, 31).

The mis-calling SM cases have induced a rising enthusiasm to the possible successful use of ICC and molecular tests on FNAC samples, although many other reports and conferences did not provide any recommendation for ICC in the SM category mainly due to the possibility of false-positive or false-negative immunocytochemical results. In fact, in the present paper, we have found four false-negative malignant cases and recently Cochand-Priollet *et al.* have found six false-positive LBC thyroid lesions without any false-negative case when applying an ICC panel of HBME-1 and CK19 (3, 5).

One of the drawbacks in routine use of molecular markers on FNAC remains the conventional cytological preparation requiring an adequate quantity of cells and resulting in a difficult scraping of the cells or the need for a second specific aspiration. Our goal, regarding the more detailed technical cytological aspects, was the application of ancillary techniques (both ICC and molecular analyses) on LBC. This method is feasible, highly reproducible and provides high yields with 100% informative immunocytochemical and molecular results

and, furthermore, with a complete concordance between cytology and surgical specimens (1, 2, 17, 18, 22, 23, 30).

Our diagnostic everyday approach was reflected in the application of the ICC panel for every SM case achieving a sub-classification in high- and low-risk thyroid lesions (29). This evidence also reflected the present results, where the ICC-positive panel was statistically correlated with a malignant outcome ($P=0.0007$), highlighted also by the ICC-negative panel in all the seven benign lesions and 86.6% positive malignancies (26 out of 30 cases).

The use of ICC for cytology can suggest a malignant outcome, although the low specificity for these immunomarkers cannot assure a definitive malignant diagnosis. In our four malignant cases with a discordant panel, we added a third immunomarker, specifically CK19, which resulted positive in two out of four, leading to double immunomarker positivity in 50% of these discordant lesions. We used two immunomarkers for two reasons: i) the limited amount of cytological material and also ii) because in some previous personal experiences, CK19 has also been observed in benign lesions such as lymphocytic thyroiditis and follicular adenomas. To overcome these false-negative results, we looked for the cytological application of *BRAF* molecular mutational analysis.

The diagnostic utility of the molecular testing panel or single *BRAF* analysis was strongly encouraged by Nikiforov in several papers, which provided a significant increase in the accuracy of FNAC of up to 95% (9, 10, 14). This evidence was also reflected in other authors' experiences, suggesting the use of a scoring model, including cytology and mutational analysis, for classifying correctly 91% of all samples, with increasing sensitivity from 77 to 96% and specificity from 68 to 80% (32, 33).

All this rising literature is also reflected in the revised management guidelines for patients with thyroid nodules and differentiated thyroid cancer, recently published by the American Thyroid Association (34), which suggests that the detection of a molecular panel (including *BRAF*, *RAS*, *RET/PTC* and *PAX8-PPAR γ*) in patients with indeterminate FNA cytology or SM improves the final diagnostic accuracy compared with a single marker.

Furthermore, the detection of *BRAF* mutation, through the activation of *MAPK*-kinase pathway, has been correlated with aggressive thyroid tumour features such as extra-thyroidal extension, advanced tumour stage at presentation and lymph node or distant metastases with the final impairment of the function of the sodium-iodide symporter and of other genes metabolising iodide, which can justify a more aggressive surgical approach (e.g. total thyroidectomy instead of lobectomy or frozen section) in patients with *BRAF* expression independently from the cytological category (32, 35, 36, 37, 38, 39, 40, 41, 42, 43).

All this literature concerning not only the diagnostic role of *BRAF* but its prognostic correlation with aggressiveness is of high impact in the SM group, for which a more extensive surgical approach can be considered when *BRAF* mutated cases underwent surgery, avoiding frozen section or useless lobectomy.

Our 15 SM-*BRAF* mutated cases define a 100% histological correlation with a diagnosis of thyroid carcinoma ($P=0.0353$) and we are in agreement with the low incidence of *BRAF* mutation in the FVPC histotypes (10%). In agreement with the XING report, our prognostic significance of *BRAF* was linked with 39% multifocal cancers and 32% extra-thyroidal and nodal-positive cases (37, 43).

The same diagnostic and prognostic association of *BRAF* expression was pointed out in the 'positive for malignancy' category with the same significant correlation ($P=0.0009$) with papillary cancer histotype and a clear correlation between *BRAF* expression and all the three aggressive parameters as lymph node metastases ($P<0.0001$), extra-capsular invasion ($P=0.03$) and multifocality ($P=0.0003$).

To our knowledge, this is the first study aimed at the introduction of a preoperative cytological approach with the sequential application of both ICC and *BRAF* molecular tests in the category of SM on LBC. The positivity of ICC should be supported at least by two immunomarkers and although it has good 86.6% sensitivity and acceptable 63.6% negative predictive value, a collegial decision concerning the clinical management of these patients is advisable. Eventually, in our setting, patients with positive *BRAF* evidence, based on the high (100%) specificity of this technique and independently from the category to which they belong to, should be given total thyroidectomies with neck dissection, meaning a consequent reduction in the incidence of second surgery or intra-operative pathology in more than 40% of cases, and including specific prognostic and predictive information as well as selecting possible future specific targeting therapies (30, 43, 44).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

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Received 8 January 2013

Revised version received 12 March 2013

Accepted 19 March 2013