Comparison of Two Rapid Assays for the Detection of BRAF V600 Mutations in Metastatic Melanoma including Positive Sentinel Lymph Nodes

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Abstract: Testing for the BRAF mutation is mandatory for the management of patients with locally advanced or metastatic melanoma. Molecular analysis based on DNA sequencing remains the gold-standard method for the screening of the different BRAF mutations. These methods must be rapid, sensitive, and specific enough to allow optimal therapeutic management in daily practice and also to include patients in clinical trials. Here, we compared the Idylla BRAF Mutation Test and the anti-BRAF V600E (clone VE1) immunohistochemistry (IHC) in 90 melanoma samples, with a focus on a challenging cohort of 32 positive sentinel lymph nodes. The BRAF status was assessed with both methods independently of the percentage of tumor cells. The concordance rate was calculated excluding both non-contributory analyses and BRAF V600K/R/M mutants due to the specific V600E-IHC test design. The incidence of the BRAF V600E mutation was 33% with both BRAF Idylla and BRAF IHC. The agreement rate was 91% (72/79). Although the agreement rate was high, we suggest that the use of IHC is more suitable for rapid BRAF testing on sentinel lymph node biopsies when associated with a low percentage and scattered tumor cells, which gave a high risk of non-contributory analysis and/or false negative results with the Idylla™ BRAF Mutation Test.

Keywords: metastatic melanoma; BRAF; RT-PCR; sentinel lymph node; immunohistochemistry

1. Introduction

Melanoma is an aggressive skin cancer with an increasing incidence worldwide [1,2]. Histopathological examination of melanoma is the gold standard for providing prognostic information according to the American Joint Committee on Cancer (AJCC) classification [3]. However, some early-stage patients still recur or metastasize [4]. Sentinel lymph node (SLN) examination helps for better staging procedure. Therefore, SLN biopsy has to be performed as a staging procedure and treatment decision in patients with tumor thickness (Breslow) ≥1 mm or ≥0.8 mm with additional risk factors [4,5]. Moreover, this procedure is
now mandatory for the new adjuvant option, allowing the inclusion of patients in different clinical trials [5].

In the last few years, new therapeutic options such as molecular-targeted therapies and immunotherapy have improved the outcomes of patients with metastatic melanoma [6]. In this regard, screening for predictive biomarkers is mandatory for the management of advanced or metastatic cutaneous melanoma, but since the establishment of different adjuvant and neoadjuvant treatments in primary tumors (pT2b-pT4) or SLN, the indications for BRAF testing are increasing considerably [3, 5]. In particular, there is an essential need to perform this testing within a short turnaround time on increasingly smaller-sized specimens.

BRAF V600 mutations are oncogenic drivers of cutaneous melanoma and are detecte

BRAF V600 mutations are oncogenic drivers of cutaneous melanoma and are detected in approximately 40% of cases [7, 8]. The most frequent mutation is a substitution of glutamic acid for valine at codon 600 (p.V600E), occurring in around 75% of the cases. Various methods for the detection of the BRAF V600E mutation have been developed, in particular targeted molecular methods based on the analysis of DNA or protein expression evaluated with immunohistochemistry (IHC) on formalin-fixed and paraffin embedded (FFPE) tissue sections or cytological specimens [9, 10]. The BRAF V600E mutation-specific antibody (clone VE1) is a monoclonal antibody specific for the mutated V600E epitope [11, 12]. This anti-BRAF V600E IHC can be easily set up in a pathology laboratory and used routinely in accordance with quality control/assurance and accreditation procedures [13]. DNA-based analyses usually require a dedicated space to set up the equipment, to avoid contamination, and need highly qualified staff and a mastered turnaround time [14]. The Idylla™ platform (Biocartis, Mechelen, Belgium) is a fully automated PCR-based system designed to be easy to implement and use in a pathology laboratory. It requires little space, avoids contamination, and necessitates only a short handling time. Both the BRAF Idylla™ Mutation test and the VE1 IHC are fast, sensitive, and specific methods, while requiring limited tumor material.

We conducted a comparative study of the performance of two validated methods set up at the Laboratory of Clinical and Experimental Pathology (LCEP) (Nice, France), the BRAF VE1 IHC (accredited according to the ISO 15189 norm: www.cofrac.fr, accessed on 19 February 2022) and the BRAF Idylla Mutation test, on 90 melanoma FFPE samples, including 32 positive sentinel lymph nodes (SLN). In addition, we reviewed the literature on the performance of the BRAF Idylla test for melanoma.

2. Materials and Methods

2.1. Samples

Fifty-eight unselected FFPE melanoma tissue samples from the LCEP, Nice University Hospital, were included consecutively between January 2018 and January 2019. In addition, 32 positive SLN were included retrospectively. The percentage of tumor cells (%TC) in melanoma samples was evaluated independently by four senior pathologists (ELM, MI, SL, and VH) on routine hematoxylin-eosin-safran (HES) stained tissue sections, according to the procedures of the French association for quality assurance in pathology (AFAQAP) [15]. All patients gave their informed consent, and the study was conducted according to the Helsinki guidelines.

2.2. Assessment of the BRAF Mutation Status with the Idylla™ Method

The BRAF mutation status was assessed routinely at the LCEP on the Idylla™ platform (Biocartis, Mechelen, Belgium). The CE-IVD Idylla™ BRAF Mutation Test is a fully automated cartridge, ready-to-use, with all reagents on-board to remove paraffin, lyse the sample, extract, and amplify DNA. The tumor area was macrodissected and transferred to the cartridge (as per the manufacturer’s instructions). A systematic HES control stain after each macrodissection ensured that the tumor material was properly selected. After a 90 min run and less than 2 min hands-on time, the final report was directly available on the console. The value of Cq (quantification cycle) was determined by different parame-
ters related to the amplification curves generated by the Idylla™ Explore software. The difference between the Cq of the internal control and the variant (or ∆Cq) identified the presence or absence of a mutation with a high sensitivity, down to 1% of mutant allele (according to manufacturer’s instructions) [16,17]. The Idylla™ BRAF Mutation Test can detect 7 mutations in the BRAF gene (Table 1). As the real time PCR uses allele specific primers separated into two chambers, the result is given as “V600E/E2/D Mutation” or “V600K/R/M Mutation” or “Wild Type”.

Table 1. Overview of mutations detected with the Idylla BRAF Assay (Idylla™ platform—Biocartis, Mechelen, Belgium).

| Exon | Results Given with Idylla | Mutation Detected |
|------|--------------------------|-------------------|
| 15   | V600E/E2/D               | c.1799T > A       |
|      |                           | c.1799_1800TG > AA|
|      |                           | c.1799_1800TG > AT|
|      |                           | c.1799_1800TG > AC|
| V600K/R/M |                     | c.1798_1799GT > AA|
|      |                           | c.1798_1799GT > AG|
|      |                           | c.1798G > A       |
|      |                           | c.1799T           |

2.3. Assessment of the BRAF V600E Status with Immunohistochemistry

IHC was performed on the same FFPE block, after molecular analysis, with the BRAF V600E mutation-specific antibody (mouse monoclonal, clone VE1, prediluted, 16-min incubation, Roche Ventana, Tucson, AZ, USA) as previously described at the LCEP [18]. The protocol was applied to 3 µm tissue sections with an automated immunostainer (Ventana Benchmark Ultra; Ventana Medical Systems, Tucson, AZ, USA) using the OptiView DAB IHC Detection Kit (Ventana). All slides were reviewed independently of the clinicopathological parameters and the results of the molecular biology by one pathologist (ELM) for the purpose of the study, as reported previously [13].

2.4. Literature Search

An electronic search of the Medline database (using PubMed as search interface) was performed. We used the following medical terms and headings for the search: “BRAF”, “melanoma”, “Idylla” or “RT-PCR” and “IHC”, “V600E,” “Idylla”, or “RT-PCR”. We screened all studies comparing IHC with Idylla for the detection of BRAF V600E and Idylla with other genetic analyses for melanoma patients, published up to April 2021.

3. Results

3.1. Samples

We studied 90 melanoma samples, including 77 metastases and 13 advanced-stage primary tumors. Metastatic sites included the lymph node (n = 54), the subcutaneous area (n = 14), the lung (n = 7) and miscellaneous sites (n = 2). 60% (32/54) of the lymph node metastases were SLN. The SLN tumor burden was evaluated according to the combined Rotterdam [19] and Dewar criteria [20]. The tumor size, evaluated according to Rotterdam criteria, was <0.1 mm for 1/32 (3%) of cases, between 0.1–1 mm for 14/32 (43%) of cases, and greater than 1 mm for 17/32 (53%) of cases with a mean of 3.69 mm [0.55–14]. The microanatomical localization, evaluated according to Dewar criteria, was combined (10/32), sub capsular (8/32), multifocal (6/32), parenchymal (6/32), and extensive (2/32) (Table 2).

3.2. Molecular Testing

The Idylla molecular analysis found 57/85 (67%) wt-BRAF V600, 28/85 (33%) mutant-BRAF and 5/90 (5.5%) non-contributive cases due to insufficient DNA input (Table 2). All of the failed results were observed in SLN (5/32; 16%). In these cases, the metastatic involvement was multifocal or parenchymal according to Dewar criteria. The tumor size ranged
from approximately 10 TC (Rotterdam < 0.1 mm) to 3 mm (mean 1.16 mm [≤0.1–3 mm]) and the macrodissected surface area was from 10 to 30 mm².

Table 2. (A) Clinical and pathological characteristics of the 90 melanoma patients included in the study. (B) Focus on the metastatic SLN samples.

(A) Clinical and Pathological Characteristics

|                          | N (%) |
|--------------------------|-------|
| Age at diagnostic        | 63 (7) |
| Median                   |       |
| Range                    | 10–93 |
| Gender                   |       |
| Male                     | 42 (47) |
| Female                   | 48 (53) |
| Tissue sample            |       |
| Metastasis               | 77 (85) |
| Primary                  | 13 (15) |
| Metastatic site          |       |
| Lymph node               | 54 (70) |
| Including SLN            | 32 (60) |
| Sub-cutaneous            | 14 (18) |
| Pulmonary                | 7 (9)  |
| Other                    | 2 (3)  |
| Percentage of tumor cells (TC) | | |
| ≤1%                      | 6 (7)  |
| 1 < TC < 10%             | 12 (13) |
| 10 ≤ TC < 50%            | 15 (17) |
| ≥50                      | 57 (63) |
| Mean percentage of tumor cell in SLN | 9.3%   |

(B) Pathological Characteristics of Metastatic SLN

|                          | N (%) |
|--------------------------|-------|
| SLN                      | 90 (100) |
| SLN tumor size           |       |
| <0.1 mm                  | 1 (3)  |
| 0.1–1 mm                 | 14 (43) |
| >1 mm                    | 17 (53) |
| SLN tumor microanatomical localization | | |
| Sub-capsular             | 8 (25)  |
| Parenchymal              | 6 (19)  |
| Combined                 | 10 (31) |
| Multifocal               | 6 (19)  |
| Extensive                | 2 (6)   |

3.3. Immunohistochemistry

IHC analysis showed 29/90 (33%) BRAF V600E positive results and 59/90 (65%) negative results. All V600K/R/M mutated cases were VE1 IHC negative. VE1 IHC was not contributive (no detectable tumor cells) in 2/90 (2%) cases corresponding to SLN, the tumor burden of which was evaluated at less than 0.1 mm and between 0.1–1 mm. These two cases were also not contributive using the BRAF Idylla™ Mutation Test (due to an insufficient amount of DNA).
3.4. Performance Comparison of Both Methods

The overall agreement between IHC and Idylla for the assessment of the *BRAF* status was calculated on 79 cases, excluding the non-contributory cases and the *BRAF V600K/R/M* mutated results due to the use of the specific *BRAF V600E*-IHC test (Figure 1).

![Figure 1. Flowcharts of the results of testing for *BRAF* with IHC and molecular method.](image)

After excluding the unpaired cases (IHC or Idylla alone) and the *BRAF V600K/R/M* results due to the specific *BRAF V600E*-IHC test design, 79 cases were included in the statistical analysis.

According to these criteria, 71/79 (90%) of the cases had concordant results. Among the discordant results, we observed one Idylla *BRAF V600E/E2/D* with a negative VE1 IHC and seven Idylla wt-*BRAF V600* with a positive VE1 IHC. All VE1 IHC were reviewed as follows: four had 5%TC and the others had equal or less than 1%TC. The VE1 IHC was repeated for these eight cases, and one sample was then reclassified as negative due to non-specific melanophage staining.

After reclassification of the discordant VE1 IHC sample, the agreement was 72/79 (91%). The concordance rate was even higher for 57/58 (98%) when only the “non-sentinel” tissue specimens were analyzed. Interestingly, while the supplier recommended a minimum amount of 50% TC for the Idylla *BRAF* assay, we did not detect any false negative results for the *BRAF V600E* mutation compared to VE1 IHC when the threshold was >10% TC.

Among the discordant samples, all the six positive VE1 IHC cases were analyzed with a molecular method on another FFPE block (metastasis or primitive), resulting in a *BRAF V600* mutation in 100% of cases (Table 3).

Because of faint labelling of tumor cells, the discordant sample—VE1 IHC negative and *BRAF V600E/E2/D* Idylla positive—was tested again with *BRAF* VE1 IHC using a red chromogen (UltraView Universal Alkaline Phosphatase Red Detection Kit, Roche). Interpretation remained equivocal due to persistent weak cytoplasmic staining. NGS analysis of the same sample with the Ion AmpliSeq™ Cancer Hotspot Panel V2 using the Ion AmpliSeq™ Library Kit™ (Ion Genestudio™ S5 Thermo Fisher Scientific, Illkirch-Graffenstaden, France) was *BRAF* wild type, whereas we expected to observe a *BRAF V600D* mutation (not detected with the VE1 antibody). This discrepancy between these two molecular biology methods may be related to a difference in the sensitivity threshold (5% sensitivity for the NGS method vs. 1% for Idylla). In addition, in this case, a new subcutaneous metastasis collected after treatment with a BRAF inhibitor was analyzed by the three methods (IHC, NGS, and Idylla), and did not reveal a *BRAF V600E* mutation. Of note, the *BRAF V600* mutation status is usually consistent between primary melanomas and matched metastases, even after targeted therapy [21–23]. Overall, a false positive result with the Idylla method cannot be ruled out (Figure 2).
Table 3. Analysis of discordant results.

| Cases   | Diagnostic Location | Stage | TC % | Idylla | ΔCQ (Idylla) | BRAF VE1 IHC | Other Sample Available | TC (%) | Molecular Methods | Results   |
|---------|---------------------|-------|------|--------|-------------|-------------|-----------------------|--------|-------------------|----------|
| #2      | Metastatic melanoma | Sentinal lymph node | IIIA | 5      | Wild type   | Delayed amplification | 10.07 | Positive | 50 | Idylla and NGS and IHC | V600E    |
| #17     | Metastatic melanoma | Sentinal lymph node | IIIA | 5      | Wild type   | Delayed amplification | 8.69  | Positive | 80 | Idylla V600E/E2/D | V600E    |
| #22     | Metastatic melanoma | Lymph node | IIIA | 5      | Wild type   | Delayed amplification | 11.39 | Positive | 70 | NGS ** | V600E    |
| #23     | Metastatic melanoma | Sentinal lymph node | IIIA | ≤1     | Wild type   | No amplification | Not applicable | Positive | 30 | Idylla V600E/E2/D | V600E    |
| #27     | Metastatic melanoma | Sentinal lymph node | IIIA | 1<CT<5 | Wild type   | Delayed amplification | 10.08 | Positive | 50 | PS ** | V600E    |
| #28     | Metastatic melanoma | Sentinal lymph node | IIIA | ≤1     | Wild type   | Delayed amplification | 9.9   | Positive | 80 | PS ** | V600E    |

Abbreviations: NGS: Next generation sequencing; PS: pyrosequencing; ** NGS Ion GeneStudio™ SS Thermo Fisher Scientific, Illkirch-Graffenstaden, France—Ion AmpliSeq™ Cancer Hotspot Panel V2); ** Pyrosequencing (Qiagen, Hilden, Germany—Therascreen BRAF Pyro Kit).

Figure 2. Example of discordant results. (a–d), a micro-metastatic sentinel lymph node that expressed BRAF V600E when analyzed by IHC and was negative with the BRAF Idylla test despite delayed amplification. (e–g), a subcutaneous melanoma metastasis considered negative for IHC BRAF V600E when compared to the control. (g) (some background noise due to intracytoplasmic pigment), when stained with a red chromogen the interpretation remains equivocal (h,i) and positive with the Idylla method. (a,e) HES stain ×20; (b) Melan A IHC ×20 (Clone A103, Roche Ventana); (c,f) BRAF V600E IHC ×20 (Clone VE1, Roche Ventana); (g) BRAF V600E IHC ×40 external control showing viable tumor cells and melanophages; (h) BRAF V600E IHC ×20 (Clone VE1, Roche Ventana—Red detection kit); (i) BRAF V600E IHC ×40 external control viable tumor cells with red chromogen; (d,j) Amplification curves with the Idylla Explore tool.

3.5. Interpretation of the Results

According to the sensitivity threshold of the Idylla method, 6/85 (7%) of cases were at risk of false negative results using a molecular biology approach (defined as a %TC ≤ 1%), all in the SLN cohort. Examination of these cases with the Idylla Explore tool revealed that 3/6 showed late amplification curves with delayed Cq and a ΔCq ranging from 9.9 to 10.98. In addition, 3/6 cases could be tested on other metastases or on the primary samples with the same Idylla™ method. The results showed 2/4 mutated cases and 2/4 WT cases, i.e., a
proven risk of a false negative in 50% of the cases when the percentage of tumor cells is 1% or less (Table 4).

Table 4. Contribution of the Idylla Explore tool for cases at risk of false negative results.

| Cases | SLN | Stage | % TC | Idylla Result | Delayed Amplification | Idylla Explore Tool (ΔCQ) | IHC BRAF | Re-Test/Other Sample |
|-------|-----|-------|------|---------------|-----------------------|--------------------------|---------|---------------------|
| #3    | yes | IIIA  | 1    | Wild type     | No                    | Not applicable           | Negative | Not applicable       |
| #5    | yes | IIIA  | <1   | Wild type     | Yes                   | ΔCQ = 10.08              | Negative | Wild type (primitive)|
| #10   | yes | IIIA  | <1   | Wild type     | No                    | Not applicable           | Negative | Wild type (metastasis)|
| #11   | yes | IIIA  | <1   | Wild type     | Yes                   | ΔCQ = 10.98              | Negative | Not applicable       |
| #23   | yes | IIIA  | <1   | Wild type     | No                    | Not applicable           | Positive | BRAF V600E (primitive)|
| #28   | yes | IIIA  | <1   | Wild type     | Yes                   | ΔCQ = 9.90               | Positive | BRAF V600E (metastasis)|

4. Discussion

We compared two biomarker-screening methods, DNA- and protein-based, to assess the BRAF mutation status in a cohort of 90 melanoma samples enriched in SLN with a low TC content. Our results showed a high agreement between these two methods, and the incidence of the BRAF V600E mutation reported in our study is representative of that published in the literature [24].

In total, 16 studies [16,17,25–38] have already reported a comparison between the Idylla method and other molecular reference methods for the assessment of the BRAF V600E mutation in melanoma patients at the time of the publication (Table 5). Most of them used the CE-IVD Idylla™ BRAF Mutation Test with a concordance rate ranging from 96.2% to 100% when compared with NGS, Sanger sequencing, pyrosequencing, or digital PCR, confirming that the test is reliable.

In addition, nine previous studies reported results using the RT-PCR Idylla method and VE1 IHC for the diagnostic of BRAF mutations [28,29,33,35,39–43]. Among these, four focused on melanoma samples [28,29,33,35], the others included central nervous system tumors, colorectal cancers, ovarian tumors, hairy cell leukemia, and salivary gland tumors [39–43] (Table 6). Among melanoma samples, the concordance rate was high (89–100%), with good sensitivity (82.3–94%) and specificity (95–100%). The discordant cases were mostly due to inadequate preanalytical treatment (as decalcification) and reflect the difficulties of interpretation of VE1 IHC, especially due to the use of a chromogen not suitable for a melanocytic pathology.
Table 5. Literature review of studies on the performance of Idylla for BRAF detection in melanoma.

| Ref.         | Study                      | Gene       | Idylla Test                       | CE-IVD      | Mutation Detected for BRAF | Sample                  | Number of Samples | Type of Tumor      | Sample Origin | TAT min | Reference Method | Concordance for BRAF % | Sens % | Spe % | PPV % | NPV % |
|--------------|----------------------------|------------|----------------------------------|-------------|-----------------------------|-------------------------|--------------------|-------------------|---------------|----------|-----------------|-------------------------|--------|-------|-------|-------|
| Melchior et al. 2015 [25] | Multicenter retrospective | BRAF       | Idylla BRAF Mutation Test         | CE-IVD      | V600E/E2/D V600K/R/M        | FFPE tissue             | 139                | Melanoma          | NS            | 90       | SS, RT-PCR, ddPCR, HRM | 97.84                   | NS     | NS    | NS    | NS    |
| Janku et al. 2015 [16] | Retrospective              | BRAF       | Idylla BRAF Mutation Test         | NS          | V600E/E2/D V600K/R/M        | FFPE tissue             | 60                 | Melanoma, CRC, PTC and others | NS            | 90       | RT-PCR, NGS       | 97 (RT-PCR) 100 (NGS) | 95     | 97    | 98    | 92    |
| Janku et al. 2016 [26] | Prospective                | BRAF       | BRAF Mutation Test prototype      | RUO         | Cell-free DNA vs FFPE       | Blood                  | 90                 | CRC, Melanoma, NSCLCC and others | NS            | 90       | PCR-based method, mass spectrometry, NGS | 88     | 73    | 98    | 96    | 85    |
| Schievie et al. 2016 [17] | Multicenter Retrospective | BRAF       | Idylla BRAF Mutation Test         | CE-IVD      | V600E/E2/D V600K/R/M        | FFPE tissue             | 419                | Melanoma, PTC, CRC and others | Primary and Metastases | 90       | SS, PS, NGS       | 96.2 (SS); 97.5 (PS) | NS     | NS    | NS    | NS    |
| Harlé et al. 2016 [27] | Retrospective              | BRAF       | Idylla BRAF Mutation Test         | CE-IVD      | V600E/E2/D V600K/R/M        | FFPE tissue             | 59                 | Melanoma          | NS            | 90       | HRM, real-time PCR, NGS, IHC | NS     | 93.5  | 100   | 100   | 93.3  |
| Barel et al. 2018 [28]  | Retrospective              | NRAS-BRAF-EGFR | Idylla NRAS-BRAF-EGFR S492R Mutation Test | RUO         | V600 E/E2/D V600 K/R        | FFPE tissue             | 36                 | Melanoma          | Primary and metastases | 110      | NGS, IHC         | 97.2 (overall) | NS     | NS    | NS    | NS    |
| Bisschop et al. 2018 [29] | Retrospective              | BRAF       | Idylla BRAF Mutation Test         | CE-IVD      | V600E/E2/D V600K/R/M        | FFPE tissue             | 37                 | Melanoma          | Primary and metastases | 90       | HRM, SS, IHC, NGS | 97.3 (overall) | 100    | 0.94  | 100   | 100   |
| Long-Mira et al. 2018 [30] | Prospective                | BRAF—NRAS  | ctNRAS-BRAF Mutation Test         | RUO         | V600E/E2/D V600 K/R         | Cell-free DNA vs FFPE   | 19                 | Melanoma          | Blood          | 90       | PS, NGS          | 84                        | 80     | 89    | NS    | NS    |
| Seremet et al. 2018 [31] | Prospective short communication | BRAF-NRAS | NS                               | NS          | NS                           | cell-free DNA           | 7                  | Melanoma          | Blood          | NS        | No               | NS                        | NS     | NS    | NS    | NS    |
| Serre et al. 2018 [32]  | Prospective and retrospective | BRAF       | Idylla BRAF Mutation Test         | CE-IVD      | V600E/E2/D V600K/R/M        | FFPE tissue             | 37                 | Melanoma          | Metastases      | 90       | No               | NS                        | NS     | NS    | NS    | NS    |
### Table 5. Cont.

| Ref.                      | Study                  | Gene                | Idylla Test                       | CE-IVD     | Mutation Detected for BRAF | Sample     | Number of Samples | Type of Tumor                | Sample Origin                                      | TAT min | Reference Method       | Concordance for BRAF % | Sens % | Spe % | PPV % | NPV % |
|--------------------------|------------------------|---------------------|----------------------------------|------------|---------------------------|------------|-------------------|----------------------------|-----------------------------------------------|---------|-----------------------|------------------------|--------|-------|-------|-------|
| Vallée et al. 2019 [33]  | Prospective            | NRAS-BRAF-EGFR      | Idylla NRAS-BRAF-EGFR S492R Mutation Test | RUO        | V600 E/E2/D V600 K/R     | FFPE tissue | 65                | Melanoma                   | Primary and metastases                      | 120     | IHC, ASA, SS, ddPCR   | 92.1 (overall)         | 100    | 100   | 100   | 100   |
| Huang et al. 2019 [34]   | Retrospective          | BRAF                | Idylla NRAS-BRAF and Idylla BRAF Mutation Test | NS         | V600 E/E2/D V600 K/R     | FFPE tissue | 210               | NS                         | CRC, Melanoma, NSCLCC and others             | NS      | NS                    | NS                     | NS     | NS    | NS    | NS    |
| Bourhis et al. 2019 [35] | Retrospective          | BRAF                | Idylla BRAF Mutation Test         | CE-IVD     | V600E/E2/D V600K/R/M     | FFPE tissue and decalcified tissue | 11 samples (paired) | Melanoma, Hairy cell leukemia | Metastases | 90 | IHC | 100 (except decalcified samples) | NS | NS | NS | NS |
| Van Haele et al. 2020 [36] | Prospective            | BRAF                | Idylla BRAF Mutation Test         | CE-IVD     | V600E/E2/D V600K/R/M     | FFPE tissue and cell block | 48                | Melanoma, NSCLCC, CRC      | Metastases | 90 | NGS, Cobas | 100 (NGS) | NS | NS | NS | NS |
| Petty et al. 2020 [37]   | Retrospective          | BRAF                | Idylla BRAF Mutation Test         | NS         | V600E/E2/D V600K/R/M     | FFPE tissue and cell block | 23                | Melanoma                   | Primary and metastases                     | 90 | SS, ARMS | 100 | 100 | 100 | 100 |
| Colombino et al. 2020 [38] | Retrospective          | BRAF                | Idylla BRAF Mutation Test         | NS         | V600E/E2/D V600K/R/M     | DNA         | 319               | Melanoma                   | Primary and metastases                     | 120 | SS, PS, NGS | 98.4 (BRAF+) | NS | NS | NS | NS |
Table 6. Overview of studies combining immunohistochemistry and Idylla for BRAF evaluation.

| Reference            | Tumor Type                                      | Number of Samples | Type Antibody | BRAF Mutation | CE-IVD | Immunostaining System | Sample | Idylla Method | BRAF Mutation with Idylla | Concordance Rate (%) | IHC Sensitivity (%) | IHC Specificity (%) | PPV (%) | NPV (%) |
|----------------------|-------------------------------------------------|-------------------|---------------|---------------|---------------|------------------------|--------|---------------|--------------------------|----------------------|---------------------|----------------------|---------|---------|
| Durlewicz et al. 2020 [39] | CNS tumor                                       | 22                | Clone VE1     | BRAF V600E    | Yes           | Ventana BenchMark ULTRA stainer | FFPE tissue | Idylla BRAF mutation Test | V600E/E2/D V600K/R/M | 86                   | NS                  | NS                   | NS       | NS       |
| Sadlecki et al. 2017 [40] | Ovarian tumor                                   | 42                | Clone VE1     | BRAF V600E    | Yes           | Ventana BenchMark GX | FFPE tissue | Idylla BRAF mutation Test | V600E/E2/D V600K/R/M | 100                  | NS                  | NS                   | NS       | NS       |
| Bourhis et al. 2019 [35] | Metastatic melanoma and hairy cell leukemia    | 11                | Clone VE1     | BRAF V600E    | No            | Ventana Benchmark XT | FFPE tissue and decalcified | Idylla BRAF mutation Test | V600E/E2/D V600K/R/M | 100                  | NS                  | NS                   | NS       | NS       |
| Bisschop et al. 2018 [29] | Metastatic melanoma                            | 37                | Clone VE1     | BRAF V600E    | Yes           | Ventana BenchMark ULTRA stainer | FFPE tissue | Idylla NRAS-BRAF-EGFR S492R Mutation Test | V600 E/E2/D V600 K/R | 97.3 (overall) | 94                  | 95                   | NS       | NS       |
| Barel et al. 2018 [28] | Melanoma (metastatic and primary)             | 36                | Clone VE1     | BRAF V600E    | No            | Ventana Benchmark XT | FFPE tissue | Idylla NRAS-BRAF-EGFR S492R Mutation Test | V600 E/E2/D V600 K/R | 100                  | NS                  | NS                   | NS       | NS       |
| Colling et al. 2017 [41] | CRC                                             | 20                | Clone VE1     | BRAF V600E    | Yes           | Ventana Benchmark Immunostainer | FFPE tissue | Idylla NRAS-BRAF-EGFR S492R Mutation Test | V600 E/E2/D V600 K/R | 90                   | NS                  | NS                   | NS       | NS       |
| Vallée et al. 2019 [33] | Melanoma (metastatic and primary)             | 65                | Clone VE1     | BRAF V600E    | No            | NS          | FFPE tissue | Idylla NRAS-BRAF-EGFR S492R Mutation Test | V600 E/E2/D V600 K/R | 89 (overall) | 82.3                 | 100                  | 100                  | 93       |
| Bodnar et al. 2017 [42] | Salivary gland tumor                           | 95                | Clone VE1     | BRAF V600E    | Yes           | Ventana BenchMark GX | FFPE tissue | Idylla BRAF Mutation Test | V600E/E2/D V600K/R/M | 97                   | NS                  | NS                   | NS       | NS       |
| Cardus et al. 2019 [43] | Hairy cell leukemia and B/T cell neoplasm      | 218               | Clone VE1     | BRAF V600E    | Yes           | Ventana BenchMark ULTRA stainer | FFPE tissue and decalcified | Idylla BRAF Mutation Test | V600E/E2/D V600K/R/M | 100                  | NS                  | NS                   | NS       | NS       |
Unlike previous studies, the current study includes positive SLN biopsies with microscopic metastases where the estimation of TC for molecular analysis is challenging and responsible for an increased risk of false negatives with molecular testing methods [44–47]. Since new adjuvant treatments in completely resected stage II-IV melanoma have demonstrated a significant impact on relapse-free survival and overall survival in patients with BRAFV600-mutant melanoma [48,49], the early detection of a BRAF V600 mutation in primary tumors (pT2b-pT4) or SLN is crucial, even at an early tumor stage [4]. These new therapeutic strategies underscore the value of testing positive SLN regardless of their tumor burden. The risk of false negative could be reduced with a macrodissection step to help minimize interobserver variation when evaluating the TC content and enrich the sample with TC for molecular biology. However, it is difficult to achieve in SLN because the tumor surface area is very restricted (i.e., multifocal or dispersed distribution—not compatible with the minimum surface area to be analyzed with Idylla). Thus, macrodissection on glass slides is most often responsible for failure due to insufficient DNA input, and macrodissection on paraffin blocks gives a high degree of variation in the cellularity due to fleeting micrometastases. The results, at risk of false negatives, must be reported and a supplementary analysis must be performed on another FFPE sample; the only alternative for these small-stage tumors remains, therefore, the analysis of the primary site. This raises the problem of the tumor heterogeneity reported in different studies [50–52], although sometimes not very comparable because of the different molecular analysis tests that are performed (NGS versus targeted sequencing, sensitivity threshold...) [53–55]. Other studies suggest that the BRAF mutation status of the primary tumor is retained in metastases [56,57] and that primary and/or metastatic tissue can be used for routine mutational analysis provided that sufficient TC content is available. Thus, the consistency of BRAF mutations among primary and metastatic tumors is still being debated. Finally, the question of inter-tumor heterogeneity also exists in the presence of several synchronous primary tumors, which sometimes happens in sun-exposed patients [57].

The Idylla™ BRAF Mutation Test allows the detection of the actionable BRAF V600E/D/K/R/M mutations with a low amount of material and has good sensitivity, but is not able to make a distinction between them, which can be an obstacle to predicting the therapeutic response [58,59]. Another limitation is the impossibility of collecting DNA from the cartridge for further analysis, such as NGS. Moreover, in several cases of SLN, it cannot be performed because it cannot meet the supplier’s recommendations (especially regarding the tumor sample size and the % of TC), although our results suggest that 10% TC is appropriate for the detection of BRAF V600E (Table 7). In the present study, the 5/90 cases that remained unamplified with Idylla contained all had low melanin content. Melanin has been recognized as a PCR inhibitor [60], but it seems to have little impact on the Idylla technology [57]. Here, the lack of amplification was related rather to an insufficient intake of tissue.

At LCEP (Nice, France), we apply the established European Organisation for Research and Treatment of Cancer (EORTC) protocol for SLN [61,62], which saves unstained slides that can be used for complementary IHC, in particular VE1 IHC. With this approach, BRAF evaluation was possible on all but two samples with VE1 IHC, without a risk of false negative results as long as the pre-analytical steps are mastered. The major advantage of VE1 IHC is that it is highly suitable for small specimens, even at a single cell level, and uses minimal tissue. In addition, it is easy to implement and cost-effective. Nevertheless, its disadvantages lie in the fact that it only detects the BRAF V600E mutant. In addition, it can be more easily subjected to pre-analytical variations, inducing a risk of false negative results and some pitfalls associated with background noise or an alteration in the extent, distribution, and intensity of the staining [35,63]. The pathologist must also be well trained in the interpretation of VE1 IHC in order to avoid the risk of false positive results. A positive BRAF V600E IHC is most often strong and diffuse throughout the tumor cells [11,13,64]. Weak or melanophage related staining should be interpreted carefully as equivocal or uninterpretable and requires control with molecular biology. In pigmented tumors with
extensive melanin an AEC-type (3-amino-9 ethylcarbazole) red chromogen can also be used instead of the DAB (3,3’-Diaminobenzidine) brown chromogen to help with interpretation. The interpretation of a negative VE1 IHC may also be challenging as it is necessary to ensure that TC are present in the IHC section, especially in SLN. In any case, a negative IHC must be confirmed by molecular analysis to avoid the risk (even low) of false negative IHC results and in order to detect other BRAF V600 mutations aside from the BRAF V600E, notably the BRAF V600K mutation, for which a therapeutic response is also observed with BRAF and MEK inhibitors [48,65–67]. Rare BRAF mutations on codon 597 or 601, also reported to be moderately sensitive to BRAF and MEK inhibitors, are not detected with an allele-specific method or IHC. Next-generation sequencing can overcome this issue, and results can be discussed on specific molecular tumor boards.

Table 7. Comparison of Idylla and IHC for the detection of the BRAF V600E mutation in melanomas.

| Principles of the Technology | Idylla (Biocartis, Belgium) | IHC BRAFV600E (Clone VE1, Roche Ventana) |
|-----------------------------|-----------------------------|------------------------------------------|
| DNA, RT-PCR                 | Protein expression, Antigen-Antibody |
| Mutations                   | Detection of a Group of Mutant Only V600E/E2/D, V600K/R/M | V600E |
| Cost/Patient *              | 140 €                       | 54 €                                     |
| Duration run                | 90 mn                       | 255 mn                                   |
| Hands-on time               | 20 mn Including block selection, cutting section or macrodissection, insertion in the cartridge | 70 mn Including cutting slide, drying time, preparation of the instrument and mounting of the slide |
| Total duration time         | 110 mn                      | 325 mn                                   |
| Competence of the operator  | Not required                 | Trained technician                       |
| Ease of interpretation      | Very easy—No specific skills | Easy—Trained Pathologist                 |
| Analytical sensibility      | Very high (1%)              | Very high (single cell-level resolution) |
| Minimal amount of material  | 50% tumor cell and 250 mm³ of tissue are recommended | Few cells, methods independent of the percentage of tumor cell |
| Preanalytic parameter       | Robust (formalin fixative)   | Delicate (formalin fixative, cold ischemia) |
| Major advantage             | Easy to use                  | Single cell-level                        |
| Major limitation            | Impossibility to collect DNA from the cartridge after test completion for NGS | Limited to the detection of the BRAF V600E mutant protein |

* In our laboratory.

SLN testing represents a major burden for pathology laboratories. Interestingly, the Merlin test (SkylineDx), recently developed on the Biocartis Idylla™ molecular diagnostic platform, aims to predict patients at low risk for lymph node metastasis [68,69]. This test, which has yet to undergo clinical validation, would reduce negative SLN biopsies, which, in addition to being a benefit to the patient, would reduce the laboratory workload and allow for a comprehensive review of the remaining SLNs.

5. Conclusions

This study demonstrated that the VE1 IHC and BRAF Idylla methods are accurate and highly correlated to the detection of the BRAF V600E mutation in melanoma. IHC is often promoted as a prescreening tool, but it definitely suits small sample sizes with few TC. For metastatic SLN, we first recommend the use of VE1 IHC, which is easy to perform using the EORTC protocol. In the case of a negative VE1 IHC, a molecular analysis can be achieved immediately on the same sample if the percentage of tumor cells and tumor surface areas are sufficient to obtain enough extracted tumor DNA. The risk of false
negative results can be prevented with the selection of an adequate specimen (cellularity—melanin load—mastered fixative condition—decalcification) and the use of a molecular assay with high sensitivity. Alternatively, in the case of negative results due to a very low number of tumor cells, a liquid biopsy can now open up new promises to evaluate the BRAF status in these patients [26,30,70].

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