Diagnostic value of immunohistochemistry for the detection of the \textit{BRAF}^{V600E} mutation in primary lung adenocarcinoma Caucasian patients

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\textbf{Background:} Non-small-cell lung carcinoma (NSCLC) patients with a \textit{BRAF}^{V600E} mutation benefit from targeted therapy. The usefulness of immunohistochemistry (IHC) as an alternative approach for the detection of \textit{BRAF}^{V600E} in NSCLC patients has not been evaluated until now. This study compared the specificity and sensitivity of IHC with other methods for the detection of \textit{BRAF}^{V600E} in primary lung adenocarcinoma.

\textbf{Patients and methods:} \textit{BRAF} mutations were analysed by DNA sequencing of a Caucasian subpopulation of selected 450 of 1509 (30\%) \textit{EGFR}, \textit{KRAS}, \textit{PI3K}A, Her2 and \textit{EML4}-\textit{ALK} wild-type (wt) primary lung adenocarcinomas. Detection of the \textit{BRAF}^{V600E} mutation was carried out by IHC using the VE1 clone antibody and compared with the results of other molecular methodologies.

\textbf{Results:} Of 450 (9\%) of tumours, 40 harboured a \textit{BRAF} mutation, which corresponded to either a \textit{BRAF}^{V600E} or a non-\textit{BRAF}^{V600E} mutation in 21 of 450 (5\%) and 19 of 450 (4\%) cases, respectively. The IHC VE1 assay was positive in 19 of 21 (90\%) \textit{BRAF}^{V600E}-mutated tumours and negative in all \textit{BRAF}^{non-V600E}-mutated tumours.

\textbf{Conclusion:} IHC using the VE1 clone is a specific and sensitive method for the detection of \textit{BRAF}^{V600E} and may be an alternative to molecular biology for the detection of mutations in NSCLC.

\textbf{Key words:} \textit{BRAF}^{V600E} mutation, immunohistochemistry, lung adenocarcinoma, targeted therapy

\textbf{introduction}

Our knowledge concerning the molecular mechanisms implicated in lung cancer has expanded rapidly in the last few years [1, 2]. With this came the fast development of molecules aimed at inhibiting tumour growth through targeting specific signalling pathways that are disrupted by genomic alterations [1, 2]. In this context, the current treatment of lung cancer patients is frequently orientated toward the administration of efficient molecular-targeted therapies, since there is an urgent need to improve the pejorative prognosis of this cancer [1].

The diagnosis of lung cancer, which is asymptomatic for a long period of time, is usually made at a late stage and on patients who cannot undergo surgery. However, the overall survival (OS) of a subpopulation of advanced-stage IIIb/IV lung cancer patients can be improved by rapid administration of a targeted therapy against genomic alterations in either \textit{EGFR} or \textit{EML4}-\textit{ALK}. Moreover, additional therapies targeting other specific somatic mutations on different oncogenes are currently under development or being studied in clinical trials on lung carcinoma patients [1, 2].

A combination of morphological and molecular diagnoses has become mandatory for the stratification of lung cancer patients for the administration of targeted treatments. However, diagnosis is frequently carried out on small-sized biopsies, obtained by limited invasive procedures. Thus, the management of these specimens must be optimised and a decision tree, taking into consideration the current ancillary methods, must be outlined to select personalised targeted therapies. A great majority of these ancillary methods can be carried out on formalin-fixed paraffin-embedded biopsies,
which allows both morphological and immunohistochemical characterisation, as well as analysis of the extracted DNA.

The lung cancer-associated molecular signature is complex and is often different from one patient to another. However, the characterisation of this signature should allow lung cancer patients to benefit from rapid treatment with a targeted therapy based on the defined molecular profile. Among the targeted therapies available for the treatment of lung adenocarcinoma patients, those targeting specific EGFR or EML4-ALK genomic alterations are routinely administered in the treatment of lung cancer patients. However, only a small subset of lung cancer patients harbouring these genomic alterations benefit from these targeted therapies. Certain genomic alterations occurring in lung adenocarcinoma, such as EGFR mutations or the EML4-ALK rearrangement, can be detected both by molecular biology and by immunohistochemistry (IHC) [3–5]. The use of an antibody to detect oncogenic mutations on tumour tissue sections allows simultaneous morphological examination, in particular an estimation of the percentage of tumour cells, as well as determination of the number and intensity of stained cells. Moreover, the results of IHC can be rapidly made available, within a few hours, in routine surgical pathology laboratories. However, in this context, the sensitivity and the specificity of the IHC technique have to be strongly controlled, in particular by optimisation of the pre-analytical and analytical phases.

Among the rare lung adenocarcinoma mutations that potentially lead to the administration of a targeted therapy is the BRAFV600E mutation, which can be detected on tumour specimens [6–10]. Among the different mutations occurring in the BRAF gene, the BRAFV600E mutation is the most frequent [11]. The BRAFV600E mutation is currently detected by molecular biology approaches carried out on DNA extracted from tumour cells. However, this procedure does not allow rapid treatment due to some technical difficulties and/or a low DNA quality/quantity. Recent studies have demonstrated the possibility of detection of the BRAFV600E mutation by IHC [12–15]. Thus, the BRAFV600E mutation has been detected by IHC in thyroid carcinoma, in thyroid, melanocytic, ovarian and colorectal brain metastases and in hairy cell leukaemia [12–15]. However, the diagnostic value and the usefulness of using IHC to detect the BRAFV600E mutation in lung adenocarcinoma have not been evaluated until now.

The purpose of the study was to compare the detection of the BRAFV600E mutation by molecular biology and by IHC in a consecutive series of 450 Caucasian patients harbouring an EGFR, KRAS, PI3KA, Her2 and EML4-ALK wild-type (wt) primary lung adenocarcinoma. In this context, the performance (specificity and sensitivity) of the BRAFV600E mutation-specific antibody (VE1 clone) was assessed in comparison with analysis by sequencing.

**methods**

**patients**

The main clinical and histopathological parameters of the patients are summarised in Supplementary Table S1, available at *Annals of Oncology* online. No patient had a history of thyroid, ovarian, colorectal or melanocytic cancer (see the Supplementary data appendix, available at *Annals of Oncology* online, for further details).

**molecular biology**

EGFR (exons 18, 19, 20, and 21) and KRAS (codons 12, 13, 61) mutations were detected using either a pyrosequencing or a direct sequencing method, as previously described [16–18]. PI3KA (exons 9 and 20) and Her2 (exon 20) mutations were detected by direct sequencing, as described [19]. The ALK-EML4 rearrangement was identified by FISH analysis in EGFR, KRAS, PI3KA and Her2 wt tumours, as previously described [18]. The BRAF mutation was detected by pyrosequencing or direct sequencing in wt tumours for the different genomic alterations mentioned above, as previously described [16–18].

**immunohistochemistry**

The BRAFV600E mutation was detected by IHC in EGFR, KRAS, PI3KA, Her2 and EML4-ALK wt tumours. The specimens used were either surgically resected (n = 149) or biopsy samples (n = 301). The generation and validation of the BRAFV600E mutation-specific antibody (clone VE1) have been reported previously (see the Supplementary data appendix, available at *Annals of Oncology* online) [12–15]. A tumour was considered as unequivocally positive for VE1 immunostaining when a distinct, strong and homogenous signal was observed in the cytoplasm of all carcinoma cells. A faint diffuse staining, any type of isolated nuclear staining, weak staining of single-interspersed cells or staining of monocytes/macrophages was scored negative, according to the previously published criteria [13].

**statistical analysis**

Clinicopathological variables were investigated for association with BRAF mutational status using Fisher’s exact test or χ² test, as appropriate. The degree of agreement between the IHC and sequencing results was assessed using the Cohen’s k coefficient. The estimation of the IHC VE1 sensitivity, specificity, predictive value and likelihood ratios was determined by comparison with the sequencing results (‘index method’). Survival rates were estimated using the Kaplan–Meier method and were compared with the log-rank test to determine significance. OS was defined as the interval between the date of diagnosis and the date of death or of the last follow-up. Progression-free survival (PFS) was defined as the duration from the date of diagnosis to disease progression.

Multivariate analysis was used to assess the effect of covariates on PFS and OS and was carried out using stepwise Cox proportional hazards regression. Analyses were carried out using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL). All statistical tests were two-sided, and the significant P-value was set at 0.05.

**results**

One thousand five hundred and nine lung adenocarcinomas from a French Caucasian population were included in this study. EGFR, KRAS, PI3KCA or Her2 gene mutations were detected in 237 of 1509 (16%), 648 of 1509 (41%), 32 of 1509 (2%) and 20 of 1509 (1%) tumours, respectively. Among the 572 EGFR, KRAS, PI3KCA and Her2 wt tumours, after evaluation of tumour sample availability, 122 of 572 (21%) showed an EML4-ALK rearrangement.

A BRAF mutation was detected in 40 of 450 (9%) EGFR, KRAS, PI3KCA, Her2, and ALK wt tumours. Among the BRAF-mutated tumours, a BRAFV600E (c.1799T>A or c.1799_1800TG>AA) mutation was present in 21 of 40 (53%) cases and other BRAF mutations were present in 19 of 40
(47%) cases. The clinicopathological and molecular characteristics of the BRAFV600E-mutated patients are given in Table 1 and Supplementary Table S2, available at Annals of Oncology online. BRAFV600E-mutated tumours showed micropapillary growth in >75% of tissue sections in 9 of 21 (43%) cases, a papillary pattern in 6 of 21 (28%) cases and an acinar pattern in 6 of 21 (28%) cases (Figure 1A1 and B1) (P < 0.001; Table 2). Seventeen of twenty-one (81%) BRAFV600Emutated tumours strongly expressed the TTF1 antigen (not shown). The mutation was significantly more frequent in never smokers [9 of 47 (19%) patients] than in the current or former smokers [12 of 403 (3%) patients] (P < 0.001; Table 2). Moreover, the incidence of BRAFV600E mutation was higher in women [11 of 158 (7%) patients] than in men [10 of 292 (3%) patients] (P = 0.089, Table 2). The non-BRAFV600E mutations were significantly more frequent in early-stage tumours (16%) than in late-stage tumours (7%) (P = 0.026, Table 2). Neither age nor pTNM (tumour node metastasis) stage were significantly associated with BRAFV600E mutations. Other clinicopathological parameters, including age, sex, smoking history and histology, were not significantly correlated with non-BRAFV600E mutations (Table 2).

Worse PFS and OS were observed in the BRAFV600E-mutated population in comparison with the BRAFV600E-mutated population (supplementary Figure S2, available at Annals of Oncology online). Multivariate analysis confirmed that pTNM stage and BRAFV600E mutation were the only independent and significant factors to predict PFS (P = 0.006 and P = 0.027, respectively) and OS (P = 0.023 and P = 0.063, respectively) (Table 3).

IHC with the VE1 clone demonstrated a strong positivity in 19 of 21 (90%) tumours that harboured a BRAFV600E mutation, as demonstrated by sequencing (P < 0.0001; k = 0.95) (Figure 1A2 and B2). When compared with the sequencing results, the IHC VE1 assay revealed 90.5% sensitivity, 100% specificity, a positive predictive value of 1 and a negative predictive value of 0.99. Homogenous intracytoplasmic staining without associated nuclear staining was demonstrated in carcinoma cells only (Figure 1A2 and B2). A concordance of 100% was found for the results of the four pathologists. Tumours with BRAF mutations other than the BRAFV600E mutation (Figure 1A4 and B4) and with wt BRAF (supplementary Figure S1, available at Annals of Oncology online) were negative on immunostaining with the VE1 antibody. All BRAFV600E-mutated melanomas stained positively, whereas non-BRAFV600E-mutated melanomas stained negatively with the VE1 antibody (not shown).

discussion

This study demonstrated that IHC using the VE1 clone is a highly specific (100%) and sensitive (90.5%) method for the detection of the BRAFV600E mutation in primary lung adenocarcinomas.

The BRAFV600E mutation is detected in 1–4.9% of lung adenocarcinoma patients, being exceptionally detected in lung squamous cell carcinoma, according to the previous series [11,
This mutation is less frequent in Asian than in Caucasian patients [11, 20, 22]. The \textit{BRAF}^{V600E} mutation represents 50–57\% of the \textit{BRAF} mutations detected in lung adenocarcinomas [11, 17, 18, 20–23]. The prevalence of \textit{BRAF} mutations in the present series is somewhat higher than those reported in other studies (9\% versus 1–5\%) [20]. This can be explained by the fact that detection of \textit{BRAF} mutations was carried out only in \textit{EGFR}, \textit{KRAS}, \textit{PI3KCA}, \textit{Her2} and \textit{ALK} wt lung adenocarcinomas from Caucasian patients. Even if the number of patients harbouring a \textit{BRAF}^{V600E} mutation is low, it...
is of great interest to look for this mutation since patients having an IIIb/IV \( \text{BRAF}^{\text{V600E}} \)-mutated tumour can benefit from therapy targeting this mutation (http://clinicaltrials.gov/show/NCT01336634). In the present series, a \( \text{BRAF}^{\text{V600E}} \) mutation was detected both in the early and late stages of lung adenocarcinoma.

As previously demonstrated, the \( \text{BRAF}^{\text{V600E}} \) mutation has been detected more frequently in female lung adenocarcinoma patients [11, 20]. Moreover, the \( \text{BRAF}^{\text{V600E}} \)-mutated patients in our study were more frequent never smokers, whereas the non-\( \text{BRAF}^{\text{V600E}} \) mutations were independent of smoking history. Similarly, previous studies reported that \( \text{BRAF}^{\text{V600E}} \) mutations are more prevalent in never smokers [20, 22]. However, Marchetti et al detected the non-\( \text{BRAF}^{\text{V600E}} \) mutations only in smokers. In contrast, other studies with emphasis on \( \text{BRAF}^{\text{V600E}} \) mutation did not find this association, probably in relation to the relatively few never smokers in the analysis who exhibited this alteration [20]. Moreover, the most frequent genetic alterations having a tobacco-related carcinogenic effect in lung cancer are G→T and G→C transversions which in the \( \text{BRAF} \) gene correspond to non-\( \text{V600E} \) mutations, in contrast to the \( \text{V600E} \) mutation due to a T→A transversion [11, 24].

Histologically, we and others found that the \( \text{BRAF}^{\text{V600E}} \) mutation was preferentially associated with a predominantly micropapillary architecture [20, 25, 26]. This pattern of tumour growth was reported to be associated with tumour aggressiveness [27]. Finally, the comparative analysis of PFS and OS between the \( \text{BRAF}^{\text{V600E}} \)-mutated and the non-\( \text{BRAF}^{\text{V600E}} \)-mutated cancer patients showed worse OS and PFS in the \( \text{BRAF}^{\text{V600E}} \)-mutated population, in agreement with previously published data [20]. Conversely, no association was found between the presence of the \( \text{BRAF}^{\text{V600E}} \) mutation and the pTNM lung adenocarcinoma staging [20]. Multivariate analysis demonstrated an independent association of \( \text{BRAF}^{\text{V600E}} \) mutations with poor PFS and OS. Similar associations have been reported in patients with colorectal cancer and melanoma with \( \text{BRAF} \) mutations [28, 29]. \( \text{BRAF}^{\text{V600E}} \) mutations markedly increase the in vitro catalytic activity of \( \text{BRAF} \) gene and result in constitutive activation of mitogen activated protein kinase (MAPK) signaling [30, 31]. Although little research has focused upon tumours with non-\( \text{BRAF}^{\text{V600E}} \) mutations, there is evidence that mutations affecting other residues have markedly different signaling effects. For instance, a number of non-

### Table 2. Comparison of clinicopathological variables with \( \text{BRAF} \) mutational status in 450 patients with lung adenocarcinoma

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall (( n ) %)</th>
<th>( \text{BRAF}^{\text{V600E}} ) (( n ) %)</th>
<th>( \text{BRAF}^{\text{WT}} ) (( n ) %)</th>
<th>( P )</th>
<th>Non-( \text{BRAF}^{\text{V600E}} ) (( n ) %)</th>
<th>( \text{BRAF}^{\text{WT}} ) (( n ) %)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Overall (450)</td>
<td>21 (5%)</td>
<td>429 (95%)</td>
<td>0.254</td>
<td>19 (4%)</td>
<td>431 (96%)</td>
<td>0.198</td>
</tr>
<tr>
<td>Smoking history</td>
<td>Male</td>
<td>292 (65%)</td>
<td>10 (3%)</td>
<td>0.089</td>
<td>282 (97%)</td>
<td>14 (5%)</td>
<td>0.411</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>158 (35%)</td>
<td>11 (7%)</td>
<td>0.001</td>
<td>147 (93%)</td>
<td>5 (3%)</td>
<td>0.990</td>
</tr>
<tr>
<td>Sex</td>
<td>Never</td>
<td>47 (10%)</td>
<td>9 (19%)</td>
<td>0.380</td>
<td>38 (81%)</td>
<td>2 (4%)</td>
<td>0.408</td>
</tr>
<tr>
<td></td>
<td>Current and former</td>
<td>403 (90%)</td>
<td>12 (3%)</td>
<td>0.380</td>
<td>391 (97%)</td>
<td>17 (4%)</td>
<td>0.026</td>
</tr>
<tr>
<td>Disease stage</td>
<td>I</td>
<td>46 (10%)</td>
<td>3 (7%)</td>
<td>0.051</td>
<td>43 (93%)</td>
<td>2 (4%)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>52 (12%)</td>
<td>3 (6%)</td>
<td>0.282</td>
<td>49 (94%)</td>
<td>6 (12%)</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>99 (22%)</td>
<td>7 (7%)</td>
<td>0.086</td>
<td>92 (93%)</td>
<td>5 (5%)</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>253 (56%)</td>
<td>8 (3%)</td>
<td>0.267</td>
<td>245 (97%)</td>
<td>6 (2%)</td>
<td>0.063</td>
</tr>
</tbody>
</table>

### Table 3. Multivariate Cox regression analysis for predicting factors for the progression-free survival (PFS) and overall survival in patients with lung adenocarcinoma carrying \( \text{BRAF} \) mutations

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>Categories compared</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFS</td>
<td>pTNM stage</td>
<td>I + II versus III + IV</td>
<td>0.051</td>
<td>0.006–0.420</td>
</tr>
<tr>
<td></td>
<td>( \text{BRAF}^{\text{V600E}} ) versus non-( \text{BRAF}^{\text{V600E}} )</td>
<td>0.282</td>
<td>0.092–0.869</td>
<td>0.027</td>
</tr>
<tr>
<td>OS</td>
<td>pTNM stage</td>
<td>I + II versus III + IV</td>
<td>0.086</td>
<td>0.011–0.711</td>
</tr>
<tr>
<td></td>
<td>( \text{BRAF}^{\text{V600E}} ) versus non-( \text{BRAF}^{\text{V600E}} )</td>
<td>0.267</td>
<td>0.057–1.259</td>
<td>0.063</td>
</tr>
</tbody>
</table>

CI, confidence interval; CIR, cumulative incidence of relapse; OS, overall survival; ADC, adenocarcinoma; TNM, tumour node metastasis.
BRAFV600E mutations that have been identified in melanoma do not activate the catalytic activity of BRAF, but do result in the activation of MAP/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK) pathway [31]. Many of these non-V600E mutations demonstrate only intermediate and low kinase activity and therefore, their classification as ‘driver’ mutations remains controversial [31].

Detection of BRAFV600E mutations in non-small-cell lung carcinoma (NSCLC) is currently analysed by a molecular biology approach carried out on extracted DNA mostly obtained from formalin-fixed paraffin-embedded biopsies [32]. The ‘bioresource’ used for the detection of BRAF mutations can be largely depleted by using this molecular approach (e.g. multiple tissue sections, dissection for tumour cell enrichment), especially since the morphological and immunohistochemical studies have been previously carried out for diagnosis on the same material, in particular on bronchial immunostained with the VE1 antibody. Taken together, these results showed that IHC with the VE1 clone was able to identify a small fraction of BRAFV600E-expressing carcinoma cells, whereas analytical molecular biology for the detection of a BRAF mutation in the same tumours was negative [13, 14]. This discrepancy could be explained by DNA hyperfixation, by the presence of large necrotic tumour areas and/or by a low percentage of BRAFV600E-mutated cells inducing a decrease in the efficiency of some molecular biology techniques (such as direct sequencing), which can be less sensitive [33]. This indicates that IHC may be more sensitive than molecular biology for the detection of the BRAFV600E mutation in lung adenocarcinomas. However, in our study, of 21 (9%) BRAFV600E-mutated tumours, were negative by IHC using the VE1 clone. These two cases demonstrated a TTF-1 negative and focal CK7-positive IHC pattern. This can be explained by an alteration in the intracellular antigen during the pre-analytical step, resulting from a long time cold ischemia, hypoxia or a hyperfixation, for example. Moreover, false-negative immunostaining may be a result of the focal expression of the antigen. The sensitivity of this marker might be improved by staining of multiple blocks from large tumour specimens, although this is not feasible with small biopsies. As previously demonstrated, the present study showed that the non-BRAFV600E mutations cannot be detected with the VE1 antibody [13, 14]. In previous studies, a weak nonspecific nuclear staining was found in some wt BRAF tumours [13, 14]. None of the BRAF wt tumours tested in this series immunostained with the VE1 antibody. Taken together, these results showed that IHC is a highly specific and relatively sensitive tool for the detection of BRAFV600E mutations in primary lung adenocarcinomas.

Even if some exceptional cases of co-mutation have been described, the BRAF mutation is considered to be exclusive to lung adenocarcinoma [11, 20, 34]. In the present study, considering that the BRAF mutation is a rare and exclusive genetic event in lung cancer, we first sought to detect genomic alterations in EGFR, KRAS, PI3KCA, Her2 and ALK before looking for BRAF mutations by both molecular biology and immunohistochemical approaches. However, we consider that the IHC BRAFV600E VE1 assay may be carried out systematically before the detection of EGFR, KRAS, PI3K, Her2 and ALK mutations by molecular biology, in particular in non-smoker women with lung adenocarcinomas harbouring a predominantly micropapillary architecture. However, with careful consideration of the false-negative rate, negative cases by IHC should be additionally tested in a stepwise molecular algorithm [33].

An immunohistochemical approach for the detection of mutations in lung adenocarcinoma is certainly a complementary method to molecular biology analysis, in particular for small formalin-fixed biopsies. Indeed, IHC with mutation-specific antibodies has the advantage of detecting the mutated protein at the single-cell level and is very sensitive for the diagnosis. Thus, IHC using antibodies targeting different molecular genetic alterations may be added to the steps in evaluating molecular diagnosis [3–5, 33, 35, 36].

This study demonstrated that IHC using the VE1 antibody is a new method to specifically detect the BRAFV600E mutation in primary lung adenocarcinoma patients. The IHC VE1 assay could be of great value in situations that may be unsuitable for genetic analysis, in particular when only a few tumour cells are present and/or a large area of tumour necrosis is associated. An integrated approach combining both anti-BRAFV600E IHC and molecular genetic analysis may also increase the diagnostic accuracy of lung adenocarcinoma BRAFV600E mutation testing. Finally, IHC targeting the BRAFV600E mutation may be used as a substitute for genetic testing in selected clinical settings and/or when there is no tumour DNA available.

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disclosure
Dr vdD and Dr DC applied for a patent on the diagnostic use of the BRAFV600E mutant-specific antibody VE1. All terms are being managed by the German Cancer Research Centre in accordance with its conflict of interest policies. The remaining authors have declared no conflicts of interest.

references


