Clinical utility of immunohistochemistry using the novel anti-BRAF V600E antibody (clone RM8) for detection of the BRAF V600E mutant protein in papillary thyroid cancers

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ABSTRACT

Background: Molecular markers are gaining increasing importance as diagnostic and prognostic tools in patients with well differentiated thyroid cancers and BRAF V600E mutation has received wide attention in this regard. Aim: To evaluate the clinical value of immunohistochemistry (IHC) using anti-BRAF V600E antibody (clone RM8) for detection of the BRAF V600E mutant protein in formalin-fixed and paraffin-embedded tissues of patients with papillary thyroid carcinomas. Materials and Methods: Patients who were managed for well differentiated thyroid cancers (n = 79) during the years 2005 and 2006 were included in the study. We evaluated the fidelity of the RM8 antibody specific for the BRAF V600E and compared its detection accuracy to real time polymerase chain reaction (PCR), which was taken as the gold standard. Results: Mutant BRAF V600E antibody was studied in 79 tissue sections, out of which 21 (26.5%) had staining for BRAF V600E in >20% of the tumour cells and these were considered positive. The BRAF staining was moderate in 10 (47.6%), strong in 9 (42.5%) and very strong in 2 (9.5%) of sections stained. There was a statistically significant concordance (P = 0.000) with quantitative PCR (qPCR) for BRAF mutant taken as standard. (Kappa agreement: 0.881) Further, the receiver operating characteristics (ROC) curve showed that IHC can be used as a comparable standard to qPCR. The highest possible sensitivity of 92% and specificity of 92.6% could be achieved by considering the cytoplasmic positivity of >20% of cells with moderate to strong intensity (AUC = 0.923) Conclusion: Our study has shown that BRAF V600E IHC can be done in a conventional manner using rabbit monoclonal antibody RM8 on formalin-fixed and paraffin-embedded tissues of patients with papillary thyroid carcinomas. With a comparable diagnostic accuracy to the gold standard qPCR testing and with an added advantage of being cost effective, this technology can be considered for use as a first-line method for detection of BRAF V600E mutations, especially in resource constrained settings.

Key words: Anti-BRAF V600E antibody, BRAF V600E mutant protein, Clone RM8, Immunohistochemistry, Papillary thyroid cancers, Polymerase chain reaction

Introduction

Thyroid cancer is the most common cancer of the endocrine system, the incidence of which in India is 13,801 new cases per 100,000 as per the GLOBOCAN data. The incidence of thyroid cancers is rapidly increasing¹ with a projected prevalence of about 5539 cases in the year 2017. The major histological types of thyroid cancers are papillary, follicular, medullary and anaplastic thyroid cancers. Papillary thyroid cancers (PTC) and follicular thyroid cancers are generally well differentiated, indolent and highly curable with current treatment modalities. However a poorly differentiated thyroid cancer can progress to anaplastic thyroid cancer, which is considered to be one of the most aggressive and deadly histological types of thyroid cancers.

A number of studies have established the association of the BRAF V600E with aggressive clinic-pathological characteristics of PTC.²-⁵ A few studies, including the author’s earlier study, have further suggested that the presence of mutant BRAF V600E portended an adverse prognosis in patients with PTCs.²-⁵ BRAF testing can be done by several methods including immunohistochemistry (IHC), Sanger sequencing, quantitative polymerase chain reaction (qPCR), mass spectrometry, real time PCR and next generation sequencing. In the present study, we have evaluated the presence of mutant BRAF V600E by conventional IHC in the formalin fixed paraffin section of patients treated for thyroid cancer using a mutation specific rabbit antibody RM8 and the results were compared to results obtained using molecular testing with qPCR, widely considered to be the current gold standard.
Materials and Methods

This is a prospective laboratory study done in a cohort of 79 patients of well-differentiated thyroid cancer patients treated at a tertiary cancer center in Chennai, South India, between the years 2005 and 2006. The clinico-pathological characteristics were retrieved retrospectively from patient case records. Further, the presence of mutant BRAF V600E protein was evaluated by IHC in the formalin fixed paraffin section blocks and the results were compared to BRAF V600E testing mutation specific qPCR.

Sample Preparation for IHC and qPCR

All the tissues were assessed by hematoxylene and eosin staining to ensure the presence of tumour and were evaluated by qualified surgical pathologist blinded to the results of the qPCR. Ten 5 μM sections were cut, out of which one section was used for BRAF V600E staining. DNA was isolated from the FFPE tumour tissue using High Pure FFPE DNA isolation kit (Roche Diagnostics GmBH, Mannheim, Germany). DNA was quantitated in Biophotometer (Eppendorf, Germany) and stored in −40°C until use.

IHC for mutant BRAF V600E

We have evaluated the antibody (Rabbit Monoclonal, clone RM8, Biorbyt Inc.) for mutant BRAF V600E protein detection in 79 tissue specimens of well differentiated thyroid cancer for the first time. Clone RM8 has been raised against mutant BRAF using the peptide specific for the mutant V600E as an immunogen and is reported to have no cross reactivity with wild type BRAF. Briefly, the slides were de-paraffinised, dehydrated using alcohol, after quenching of the endogenous peroxidase; the antigen was retrieved by wet autoclaving for 10 min in citrate buffer pH 6.0. The slides were stained using the secondary antibody and developed using chromogen DAB. The slides were counterstained using hematoxylene mounted using the DPX mountant.

IHC scoring

The tumour cells were scored by a qualified onco-pathologist who was blinded to the PCR results of identifying the mutant BRAFTV600E. BRAF RM8 immunoreactivity was scored for both intensity (0–3+) and percentage of immunoreactive tumour cells (0-100%). We followed “lenient” criteria where any cytoplasmic positivity was considered positive and “stringent” criteria, tumor cells with cytoplasmic activity in 20% and above being considered positive. We scored samples as IHC positive only if the intensity of the staining was 2+ or 3+ as shown in Figure 1. We optimized the staining using the antibody on melanoma sections known to harbor BRAFTV600E mutation as shown by qPCR and this was used as a positive control.

Molecular Testing for BRAF Status by qPCR

BRAF gene mutation analysis was done using BRAF mutation detection kit (Helini Biomolecules, India) using real time PCR. Performance characteristics of the kit was validated using thyroid cancer cell lines, (T238, SW1736, N-Thy, Calc62, BCPAP, OGK-M, Hth7). The test uses allele specific probes to identify the presence or absence of mutations V600E (Val600Glu), V600K (Val600Lys), V600D (Val600Asp), V600R (Val600Arg) and V600M (Val600Met) found in codon 600 of BRAF gene by real time PCR (ABI 7500, Applied Biosystems) with a detection sensitivity of 1% mutant in the background of wild type genomic DNA.

Results

The percentage of tumor cells staining and staining intensity for BRAF V600E was evaluated and is depicted in Table 1. We considered cutoff of BRAF V600E in more than 20% of the tumor cells as positive for the presence of the mutation. The criteria followed were based on criteria for BRAF V600E staining using the widely used antibody, VE1. Scoring the tissue sample as positive based on any detectable cytoplasmic staining was termed “lenient” scoring; this however showed a higher discrepancy with the gold standard qPCR results [Table 2]. Scoring of the positive BRAF V600E based on greater than 20% of tumor cells, called the “stringent” mode was correlating well with qPCR.

BRAF V600E expression in thyroid cancer

BRAF V600E was considered negative in 73.4% (58/79) patients and positive in 26.5% (21/79) patients based on the

<table>
<thead>
<tr>
<th>Category</th>
<th>Percentage of tumor cell positivity</th>
<th>(n=79)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Absent</td>
<td>45 (57)</td>
</tr>
<tr>
<td>2</td>
<td>&lt;20%</td>
<td>13 (16.5)</td>
</tr>
<tr>
<td>3</td>
<td>20–50</td>
<td>10 (47.6)</td>
</tr>
<tr>
<td>4</td>
<td>51–75</td>
<td>9 (42.8)</td>
</tr>
<tr>
<td>5</td>
<td>&gt;75</td>
<td>2 (9.52)</td>
</tr>
</tbody>
</table>

IHC: Immunohistochemistry

Figure 1: Immunohistochemistry using the novel anti-BRAFV600E antibody (clone RM8) showing strong cytoplasmic positivity for detection of the BRAF V600E mutant protein in papillary thyroid cancers
above mentioned criteria. Table 1 shows the percentage of tumor cell positivity. BRAF V600E was negative in 45/79 (56.9%). Focal positivity with staining in <20% of tumour cells was found in 16.4% (13/79) BRAF V600E positive staining in 20-50% of the tumour cells was found in 10/21 (47.6%), 51-75% positivity in 9/21 (42.8%) and >75% tumour cell positivity was observed in 2/21 (9.5%). Conventional papillary thyroid cancer patients showed a higher frequency of BRAF V600E mutations in this group. (23/53; 43.4%) (data not shown)

Detection of the presence of BRAF V600E by IHC correlates significantly with the mutation specific qPCR

Among the patient samples showing any positivity for BRAF V600E by IHC (n = 27), 92% (23/27) were found to be positive by qPCR based detection and 92.6% (50/52) were negative for BRAF V600E by both the tests, namely IHC and qPCR. This correlation was statistically significant. The correlation of the two tests were found to be statistically significant (P = 0.000) as shown in Table 3.

Development of the scoring system of BRAF V600E for diagnostic testing in clinical samples

We compared the diagnostic accuracy of two different scoring patterns of IHC namely the “lenient” and “stringent” with qPCR evaluating the sensitivity, specificity, positive predictive value, negative predictive value. We found that “stringent” scoring patterns where we considered more than 20% of the tumor cells expressing the presence of BRAF V600E as positive performed with better diagnostic accuracy, correlating better with the qPCR technology [Table 4]. The receiver operating characteristics (ROC) curves showed that IHC can be used as a comparable standard to the qPCR, the highest possible sensitivity of 92% and specificity of 92.6% could be achieved by considering the cytoplasmic positivity of >20% of cells with moderate to strong intensity. (ROC = 0.923, p=0.000) [Figure 2].

Discussion

The dawn of the present century has marked a renewed interest in exploring the role of mutant BRAF in the pathogenesis of many cancers. Mutations in the BRAF gene have been reported in about 7%-15% of all cancers, the highest provenances being reported in melanomas, (40%-70%). Over the years, numerous studies have shown a high prevalence of BRAF V600E mutations in thyroid cancer and currently BRAF V600E mutation is one of the most prevalent and widely studied molecular event in the pathogenesis of adult PTCs.

The optimal method for BRAF mutation detection remains to be determined despite advances in molecular detection techniques. It is noteworthy to mention that each of the methods has its own sensitivity, specificity, costs and turnaround times. Many authors consider the various DNA based molecular assays (sanger sequencing, PCR, mass spectrometry, real time PCR and next generation sequencing) to be a gold standard method to assess the mutant BRAF status. A recent study done of molecular diagnostics of BRAF V600E have shown that qPCR specific for mutant had the highest detection levels compared to Sanger sequencing qPCR was hence used as a gold standard in our present study.

Molecular methods are costly, at times time consuming and not routinely available in all diagnostic laboratories. Moreover, some samples may not be suitable for molecular testing because of their inadequate tumor content, improper fixation and the variable quality of DNA extracted, especially when attempted from archival blocks. Thus, there are occasional instances where in alternative diagnostic BRAF mutation detection platforms need to be explored, and IHC promises to be one such alternative.

Table 2: Correlation of qPCR for detection of BRAF V600E versus detection by IHC

<table>
<thead>
<tr>
<th>BRAF Status</th>
<th>IHC negative</th>
<th>IHC&lt;20%</th>
<th>IHC 20–50%</th>
<th>IHC 51–75%</th>
<th>IHC&gt;75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=25)</td>
<td>2 (8)</td>
<td>3 (12)</td>
<td>10 (40)</td>
<td>8 (32)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>(n= 54)</td>
<td>43 (79.6)</td>
<td>10 (18.5)</td>
<td>0</td>
<td>1 (1.9)</td>
<td>0</td>
</tr>
</tbody>
</table>

P=0.000. Chi square=55.387. IHC: Immunohistochemistry, qPCR: Quantitative polymerase chain reaction

Table 3: IHCs versus qPCR

<table>
<thead>
<tr>
<th>BRAF V600E qPCR</th>
<th>BRAF V600E IHC +</th>
<th>BRAF V600E IHC –ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (n=25)</td>
<td>23 (92)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Wild Type (n = 54)</td>
<td>4 (7.4)</td>
<td>50 (92.6)</td>
</tr>
</tbody>
</table>

P=0.000. IHC: Immunohistochemistry, qPCR: Quantitative polymerase chain reaction
IHC is a well-established and routinely used technique in most of the pathology laboratories across India and has the added advantage to provide the pathologist and clinician a rapid and inexpensive test result. More recently, in the era of personalized medicine the utility and importance of IHC seems to be increasing and its role in BRAF V600E mutations in thyroid cancers is being explored. A mutation-specific mouse monoclonal antibody (clone VE1), which specifically detects BRAF V600E mutated protein, has been developed and it is now commercially available.\(^{10,11}\) It has been shown in various studies that IHC with this antibody is sensitive and specific for the detection of the mutant BRAF in malignant melanomas.\(^{12,13}\) The use of VE1 antibody for the detection of mutant BRAF V600E in papillary thyroid cancer tissues has also been explored in various studies with promising results. Many other studies have reported that the use of IHC for the detection of mutant BRAF in PTCs is a reliable method with a high specificity (93–100%) sensitivity (85–100%) positive and negative predictive value.\(^{10,11,14-18}\)

However, VE1 should ideally be used on the Ventana BenchMark Ultra platform (Ventana Medical Systems Inc.) and this platform may not be available with many low resource centres including ours. Testing on the Ventana BenchMark XT platform is more expensive with cost per slide being higher compared to the conventional one. Here we have compared the performance of another mutation V600E specific clone RM8 and correlated the results with that of mutation specific qPCR, and found the correlation to be statistically significant.

In clinical practice, several authors have explored the option of subjecting patients with suspected malignant thyroid nodules to a preoperative BRAF V600E mutation testing along with other molecular markers in an attempt to guide the initial surgical treatment in PTC.\(^{19,20}\) It is therefore prudent that the treating clinician is aware of the various platforms for detection of mutant BRAF. A qPCR based mutant BRAF detection can be easily done on a wide variety of specimens including FNAC specimens from suspicious thyroid nodules, thus giving an additional diagnostic potential of this technology. Further, IHC based detection using a mutation specific antibody can be applied only in thyroid cancers as only BRAF V600E mutation is frequently observed and not V600K, V600D, V600R and BRAF wild type. However, from the perspective of cost effectiveness, the IHC technology seems to score over qPCR and the other DNA based molecular methods. In summary, although a qPCR based testing may be little more expensive than IHC based testing, DNA based molecular tests are more objective and accurate and further it can detect other BRAF mutants as well and this can potentially have a clinical application in several other cancers with a mutant BRAF.

However, despite the mounting evidence, the most recent American Thyroid Association guidelines does not allow for a routine testing of mutant BRAF V600E in the initial risk stratification of patients with well differentiated thyroid cancers.\(^{21}\) However, guideline does}
recommend testing for BRAF V600E and/or TERT mutation status in the ongoing dynamic risk assessment of relapse.\textsuperscript{[21]} There seems to be a growing consensus that BRAF V600E mutational status should be studied in association with other molecular and clinico-pathological prognostic factors for a better risk stratification and this field is evolving.\textsuperscript{[22-24]}

The main limitation of our study was the modest numbers ($n = 79$) precluding us from making any firm recommendations. The results of our study with regards to the use of the novel RM8 antibody specific for the BRAF V600E mutations needs to be confirmed in a study with larger cohort of patients with PTCs. Further it remains to be seen as to how BRAF IHC compares with superior technologies like the next-gen sequencing (NGS). An NGS platform has the capability of providing further information about the actual proportion of cells carrying a given mutation and this could further help to better understand the clonal composition of the tumor. Furthermore, NGS technology can assess additional genes and this capability could aid in better diagnoses and prognostication of patients with PTCs.

**Conclusion**

Our study has shown that BRAF V600E IHC can be done in a conventional manner using rabbit monoclonal antibody RM8 on formalin-fixed paraffin-embedded tissue of patients of papillary thyroid carcinomas. With a comparable diagnostic accuracy to the gold standard qPCR testing and with an added advantage of being cost effective, this technology can be considered for use as a first-line method for detection of BRAF V600E mutations. Further, a combination of IHC and molecular approaches in selected cases (negative or uninterpretable cases) can possibly improve the sensitivity of detection of mutant BRAF in thyroid cancers, especially in resource constrained settings. We hope that this information will help the clinicians to better understand, manage and prognosticate patients with well differentiated thyroid cancers.

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