

IMMUNOHISTOCHEMICAL EXPRESSION OF BRAFV600E IN MELANOMA CASES

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ABSTRACT

Objective: To evaluate the diagnostic value of Immunohistochemistry (IHC) for finding BRAFV600E mutation in melanoma patients.

Study Design: Cross-sectional study.

Place and Duration of Study: Department of Histopathology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, Jan 2018 to Apr 2019.

Methodology: Hundred histologically confirmed cases of melanoma aged between 18-80 years were included over a period of one year. To create the tissue microarray and to conduct immunohistochemistry with VE1 antibody melanoma lesion samples were selected.

Results: Ninety-four cases out of 100, were melanoma positive for BRAFV600E detected on immunohistochemistry (IHC) using a monoclonal antibody. The skin was the primary site in seventy-nine (79%) patients, unknown (metastatic) in nineteen and uveal was only in two cases. The diagnostic significance of immunohistochemistry (IHC) for detecting the BRAFV600E mutation in melanoma patients was independent of age, site and stage at diagnosis (p -value <0.05).

Conclusion: Testing of BRAFV600E mutation by immunohistochemistry is a rapid, reliable and cost-effective tool in melanoma patients which may further help clinicians in initiating targeted therapy for these patients.

Keywords: BRAFV600E mutation, Immunohistochemistry, Monoclonal antibody, Malignant melanoma.

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INTRODUCTION

The worldwide incidence of melanoma has steadily increased over the last several decades. The prevalence varies by race: 1.9/100,000 in Hispanics 9.2/100,000 in whites, and 0.7% to 1.2/100,000 in blacks and Asian¹. In spite of significant advancements in medicine, melanoma remains a huge burden on the healthcare system in developing countries due to the substantial annual costs incurred for its management².

Melanoma is a difficult carcinoma to cure ranking the fifth and seventh most frequent tumour diagnosed in males and females respectively³. In malignant melanoma, activating BRAF mutations are identified at a high rate. More than 90% of such mutations are characterized by glutamate to valine replacement at location 600 (V600E), leading to a substantial rise in protein kinase activity. Especially in primary and metastatic melanoma (20-70%), papillary thyroid tumour (40-70%), colorectal cancer (5-10%), and selected benign tumours like melanocytic nevi⁴, these mutations are seen at increased levels. Because of its incidence and

contribution in raising tumour growth and metastases, BRAFV600E has been recognized as an effective biomarker for clinicians⁵.

DNA based techniques like direct DNA sequencing and allele-specific PCR are the gold standard to detect BRAF mutations in clinical settings. The most common ones are the amplification refractory mutation system-PCR, classical Sanger sequencing, high-resolution melting, ligase detection reaction, pyrosequencing MassArray and next-generation sequencing (NGS)⁶.

Though these techniques for mutational analysis are accurate and reliable, they are expensive and time-consuming. The genetic analysis becomes less feasible because of its relatively high labour cost, costly equipment, technological flaws, complex analysis and the need for a genetic testing specialist and often not regularly accessible in all anatomic pathology laboratories⁷.

The production of new antibodies directed against V600E antigen has lately, unlocked the way to a simpler but more successful technique for the detection of this mutation and now is available commercially from Spring Bioscience and Ventana^{8,9}. The first BRAFV600E -specific antibody (VE1) was developed

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by Feller *et al*, which has validated to be very sensitive and precise for recognizing this alteration in melanoma cases¹⁰ and have paved the way to use IHC, which is a relatively quicker, less costly and more accessible technique for evaluating the BRAF status in the biopsy specimen.

In view of the above, this study was designed to validate the diagnostic significance of IHC in detecting the BRAFV600E mutation in melanoma patients. Currently, the study is prospective using a relatively larger sample size which would be a valuable contribution to the already existing pool of knowledge.

METHODOLOGY

The cross sectional study was approved by the institutional review board of Armed Forces Institute of Pathology [FC-READ-IRB/18/885] from January 2018 to April 2019.

Inclusion Criteria: The histologically confirmed cases of melanoma aged between 18-85 years of either gender were included using non-probability consecutive sampling technique.

Exclusion Criteria: The patients having prior chemotherapy, antiangiogenic therapy and radiotherapy were excluded.

The sample size was calculated by using OpenEpi calculator by taking the prevalence of BRAFV 600E mutation on immunohistochemistry in melanoma patients i.e. 94%⁸, margin of error=4%, confidence level=95%, then calculated sample size was 100. Clinicopathological features such as gender, age, site, stage, distant and nodal metastasis were noted.

Four-micrometer thick slices were cut on microtome from formalin-fixed, paraffin-embedded (FFPE) tissue blocks. Testing was performed with the Benchmark ULTRA platform with pre-peroxidase inhibition and primary antibody incubation for 15 min at 37°C using anti V600 E monoclonal primary antibody (Ventana Medical Systems, Inc Cat. Number 790-4855). Anti-body's final concentration was ~12 µg/ml. To visualize the BRAF expression diaminoben-zidine staining was used. Hematoxylin II and Bluing Reagent were used for counter staining the tissues for 4 min. Negative control in the form of mouse monoclonal antibody was also applied.

Ultimately, specimens after immunohisto-chemistry were seen by autonomous double-blinded analysts who didn't have cellular and laboratory-based patient's records. In most cases, the analyses of the two observers were comparable and re-examination as well

as consensus resolved few disparities. In positive tumor cells, the VE1 antibody demonstrates cytoplasmic staining in accordance with Boursault *et al*¹¹. Immunostaining was generally defined as positive or negative. When >90% of cancer cells displayed distinct moderate to strong brown intracellular discoloration they were taken as positive, whereas when there was no discoloration, only nuclear stain, focal staining of tumor cell-sor staining of lymphocytes, monocytes/macrophage they were considered negative. Furthermore, the immunostaining intensity was graded 0 if no discoloration was observed, Grade 1 if there was weak cytoplasmic discoloration, moderate diffuse cytoplasmic staining was given grade 2 and grade 3 was allotted with strong mainly granular intracellular brown discoloration. No staining (grade 0) and grade 1 staining were considered negative for V600E, whereas grade 2 and grade 3 were considered as positive as per Løes *et al*¹².

Statistical software SPSS-25 was used to analyze the data. Median (range) was computed for quantitative variables like age as the assumption of normality was violated. Frequencies and percentages were computed for qualitative data like gender, site, stage, distant and nodal metastasis along with outcome variables i.e. BRAFV600E detected by immunohistochemistry using a monoclonal antibody in melanoma patients. Effect modifiers were controlled through the stratification of age, gender, site, stage, distant and nodal metastasis. chi-square test was applied post stratification. The *p*-value of <0.05 was considered statistically significant.

RESULTS

Out of the total hundred patient samples range for age in this research was 18-80 years with a median age of 63 (24-85). Fifty-nine (59%) were male and 41 (31%) were female. The skin was the primary site in seventy-nine (79%) patients, unknown (metastatic) in nineteen (19%) and uveal was only in two (2%) cases. Seventeen (17%) of the patients had stage I disease at diagnosis, thirty-four (34%) patients were at stage II, forty-three (43%) were at stage III and six (6%) were at stage IV. According to the melanoma staging system of the American Joint Committee on Cancer (AJCC), twenty-three (23%) patients had M1a disease, nineteen (19%) patients had M1b disease and fifty-eight (58%) patients had M1c stage, shown in the Table-I.

Ninety four cases of Melanoma were positive for BRAF V600E detected on immunohistochemical (IHC) method using a monoclonal antibody, shown in Table II.

Table-I: Demographic details of the patients.

Parameters	n (%)
Gender	
Male	59 (59%)
Female	41 (31%)
Site of Melanoma	
Skin	79 (79%)
Unknown	19 (19%)
Uveal	02 (2%)
Stage at Diagnosis	
I	17 (17%)
II	34 (34%)
III	43 (43%)
IV	6 (6%)
Distant Metastasis	
M1a	23 (23%)
M1b	19 (19%)
M1c	58 (58%)
Nodal Metastasis	
<2	49 (49%)
>2	51 (51%)

Table-II: Frequency of BRAF V600E detected on immunohistochemical (IHC) method using amonoclonal antibody in melanoma patients.

BRAF V600E on IHC	n (%)
Positive	94 (94%)
Negative	06 (6%)

Table-III: Association between clinicopathological features and frequency of BRAFV600E detected immunohistochemical method using monoclonal antibody in melanoma patients.

Demographic Details	BRAF V600E on Immunohistochemistry		p-value
	Positive, n(%)	Negative, n(%)	
Age			
18-40 years	08 (8%)	04 (4%)	0.002
40 years	86 (86%)	02 (2%)	
Gender			
Male	57 (57%)	02 (2%)	0.224
Female	37 (37%)	04 (4%)	
Site of Melanoma			
Skin	75 (75%)	04 (4%)	0.03
Unknown	18 (18%)	01 (1%)	
Uveal	01 (1%)	01 (1%)	
Stage at Diagnosis			
I	16 (16%)	01 (1%)	<0.001
II	33 (33%)	01 (1%)	
III	40 (40%)	03 (3%)	
IV	05 (5%)	01 (1%)	
Distant Metastasis			
M1a	21 (21%)	02 (2%)	0.825
M1b	18 (18%)	01 (1%)	
M1c	55 (55%)	03 (3%)	
Nodal Metastasis			
<2	47 (47%)	02 (2%)	0.678
>2	47 (47%)	04 (4%)	

Multivariate analysis showed that the age, site of Melanoma and stage at diagnosis are independent

factors (p -value <0.05). However, the same may be affected with gender, distant and nodal metastasis, shown in the the Table-III.

DISCUSSION

In the era of personalized medicine use of immunohistochemistry as a prognostic and predictive marker is on the rise, since it is a comparatively quick and economical method that doesn't necessitate large tumour cell substance¹³. It is also simple to perform and is regarded to be extremely sensitive and precise as the monoclonal antibodies of a specific genetic alteration are typically focused towards the epitope of beneficial concern¹⁴.

Regarding melanoma, few studies are available on the comparison of the diagnostic utility of clone VE1 in detecting BRAF V600E mutation with those of other molecular methodologies. Moreover, the clinical characteristics of patients with melanoma BRAFV600E mutation are not consistent across different studies^{15,16}. The emphasis of this research wasn't on assessing the incidence of BRAFV600E mutations in melanoma, but to discover the likelihood of employing a monoclonal VE1 antibody immunohistochemistry test as a substitute for currently used molecular methodologies in identifying BRAF V600E mutation in melanoma. Hence, our cases were not chosen particularly to validate mutation rates.

We found BRAF V600E mutations were positive in 94% of the patients. Our results are almost like those of Schirosi *et al*¹⁷, who studied 64 histologically confirmed metastatic cases using the fully automatized immuno-histochemistry method, reached a positive predictive value (PPV) of 97.1%. Similarly, our results are also the same as that of Yaman *et al*¹⁷, who studied 48 specimens including both the primary and metastatic melanoma biopsies using the fully automatized immunohistochemical technique with supplementation of an amplification reagent kit, reported a positive predictive value of 98.2% & negative predictive value of 89.7%. Another study close to our results is Haung *et al*¹⁸ who in a study of 73 cases of melanoma from lymph nodes and metastatic sites using the bond max auto-stainer showed the sensitivity and specificity of 100% and 98.6% respectively with an overall concordance of 96% between immuno-histochemical and direct sequencing method. Similarly, Manfredi *et al*. Have mentioned highly concordant results between immunohistochemistry and molecular testing of mutated BRAFV600E in primary and metastatic melanoma¹⁹.

It should, however, be acknowledged that our observed prevalence of BRAFV600E in melanoma is significant, which is a precedent. Researches on the incidence of BRAF mutations in melanoma signify that they're often seen in melanomas of skin with a comparatively high prevalence observed in those who are sporadically subjected to sunlight versus continuously subjected to sunlight⁶. As a greater percentage of our cases were from continuously sun-exposed sites, this could account for the higher frequency of BRAFV 600E throughout the current cohort. The results of this study, therefore, shed light on the possible use of IHC with the anti-BRAF (V600E) antibody as an additional evaluation tool for assessing the status of BRAFV600E mutation in melanoma.

With regards to the association between clinicopathological features and immunohistochemistry results, age, site and stage at the time of diagnosis were significantly associated with BRAFV600E mutation. Our findings are consistent with literature reporting that the BRAF mutation has been correlated with younger patients²⁰. Additional studies on a larger cohort, multi-centre, standardized and prospective studies are essential to authenticate our results and for immunohistochemistry to supplement or substitute DNA-based techniques.

CONCLUSION

The findings of our study allow the use of immunohistochemistry as a cost-effective, rapid and reliable diagnostic tool in detecting the BRAFV600E mutations in melanoma patients. This would help the clinicians in initiating the personalized medicine tailored to the needs of individual patients timely. However, more studies with a larger sample size are needed in order to draw a robust conclusion regarding the efficacy of immunohistochemistry in detecting the BRAFV600E mutations in melanoma patients.

Conflict of Interest: None.

Authors' Contribution

MUC: Direct contribution, conception, data analysis. FA: Manuscript preparation. NJ: Data analysis. RA: Intellectual contribution to the analysis. SB: Data collection. HM: Intellectual contribution to interpretation.

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