

# The Immunoreactivity Patterns of BRAF VE1 and its Diagnostic Value in Brain Tumors

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## Research Article

**Keywords:** brain tumors, BRAF-V600E, VE1, immunoreactivity patterns, pathological diagnosis

**Posted Date:** May 4th, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-46822/v2>

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# Abstract

## Background

BRAF-V600E mutation is considered as a material biomarker with diagnosis, prognosis and prediction in brain tumors. At present, molecular detection remains the most responsible approach for BRAF V600E mutation. The accuracy of Immunohistochemical test for Anti-BRAF VE1 antibody has rarely been systematically evaluated in brain tumors, despite it is widely used in pathological diagnostic practice.

## Methods

A total of 120 brain tumors were detected by immunohistochemical staining and real-time qPCR for BRAF V600E mutation. These cases were divided into 4 groups by Immunoreactivity Patterns under a microscope, including: diffuse positivity, reliable positivity of single tumor cells, uniform "coating" positivity and Negative. Then the Image-Pro Plus 6.0 image processing and analysis software were used to measure the stained area, integrating absorbance(IA)of stained area. The accuracy of immunohistochemical detection was verified by real-time qPCR. The BRAFV600E mutation prediction model was further established using Bayesian classification with tumor type, area of positive and average IA of stained area. All statistical analyses were performed in R (version R 3.6.3)

## Result

The sensitivity of immunohistochemical detection was 97.01% and the specificity was 100%( $P < 0.0001$ ). The BRAFV600E mutation prediction model was further established with a AUC value of 0.95.

## Conclusions

BRAF VE1 staining could replace molecular detection to some extent, on the premise of mastering the key points in the interpretation of BRAF VE1 immunostaining. Otherwise, the prediction model of BRAF V600E mutation may provide a possible scheme for artificial intelligence interpreting of immunohistochemical staining of BRAF VE1.

## Introduction

BRAF is an oncogene, which mutation has been detected in several types of cancer, such as malignant melanoma, papillary thyroid carcinoma, colorectal cancer, Langerhans cell histiocytosis and brain tumors, including gangliocytoma/ganglioglioma(GC/GG), pleomorphic xanthoastrocytoma(PXA), Dysembryoplastic Neuroepithelial tumor(DNT), pilocytic astrocytoma(PA), Epithelioid glioblastoma(E-GBM) Polymorphous low-grade neuroepithelial tumor of the young(PLNTY) and Papillary craniopharyngioma(p-CPG)<sup>[1; 2; 3; 4; 5; 6]</sup>. BRAF gene, which is located on chromosome 7q34, can activate the MAP kinase/ERK signaling pathway in response to cellular growth signals. B-Raf protein is a serine/threonine kinase and a component of the receptor tyrosine kinase (RTK) signaling pathway<sup>[7]</sup>. The majority of mutations on BRAF affects a mutational hot spot at amino acid position 600 and is characterized by the exchange of valine by glutamate (referred to as BRAFV600E)<sup>[7]</sup>. BRAF mutation is considered as an important biomarker with diagnostic, prognostic, and predictive potential in several clinical settings, especially in central nervous system tumors. BRAF VE1 antibody, a mouse monoclonal primary antibody, is used in the identification of the BRAF V600E mutant protein. Previous studies have verified the sensitivity and specificity of anti-BRAF VE1 antibody in melanoma<sup>[8]</sup> and papillary thyroid carcinoma<sup>[9]</sup>. However, few studies have assessed the reliability of BRAF-VE1 immunohistochemistry (IHC) in brain tumors. Breton has examined the expression of BRAF VE1 in a series of neuroepithelial neoplasias, but the sensitivity and specificity of immunohistochemical detection of BRAF-V600E mutant protein were not evaluated due to the fact that some of the tissue samples were not insufficient for further confirmation by molecular assay<sup>[10]</sup>. The aim of this study was to assess the utility of BRAF-VE1 IHC compared to molecular biology on a large series of CNS tumors, in order to provide a useful reference for the interpretation of BRAF-V600E IHC in brain tumors.

## Materials And Methods

### Subjects

Data of a series of 120 patients with brain tumors were retrospectively collected October 2016 and October 2020 at Guangdong Sanjiu Brain Hospital. Pathology reports and hematoxylin and eosin staining slides were reviewed by 2 neuropathologists according to the 2022 WHO classifications of central nervous system tumors. The clinical, histologic, the Immunoreactivity Patterns of BRAF V600E and molecular characteristics are summarized in Table 1. The study was approved by the Ethics Committee (EC number: 2020-010-087) of Guangdong Sanjiu Brain Hospital and was conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants. All methods were performed in accordance with the relevant guidelines and regulations.

Table 1  
Clinical data and BRAFV600E molecular findings in 120 cases of brain tumors

Tumor entity (N)	Age median (range)	Gender (N)	Localization	BRAF V600E(IHC)				BRAF V600 E(PCR)	
				Diffuse positive	Single positive (N)	Equivocal positive(N)	Negative (N)	Mutation (N)	Wild type (N)
GC/GG (48)	21(5-59)	M (26)	Temporal, Frontal, Occipital, Parietal, Sellar region, Thalamus,	26(54.1%)	10(20.8%)	5(10.4)	8(16.7%)	36(75%)	12(25%)
		F (22)							
DNT (17)	21(3-61)	M (11)	Temporal, Frontal, Occipital, Parietal,	2(11.8%)	0	5(29.4%)	10(58.8%)	2 (11.8%)	15(88.2%)
		F (6)							
PXA (11)	28(5-63)	M (5)	Temporal, Frontal, Occipital, Parietal	6(54.4%)	0	3(27.3)	2(18.2%)	7 (63.6%)	4(36.4%)
		F (6)							
E-GBM(12)	28(5-50)	M (4)	Temporal, Frontal, Parietal, Occipital, Sellar region	6(50%)	0	4(33.3%)	2(16.7%)	7 (58.3%)	5(41.7%)
		F (8)							
PA (13)	13(1-60)	M (9)	Cerebellum, Pineal gland, Frontal, Medulla oblongata,	1(7.8%)	0	4(30.8%)	8(61.5)	1 (7.8%)	12(92.2%)
		F (4)							
p-CPG(14)	45(29-61)	M (12)	Sellar region,	14 (100%)	0	0	0	14(100%)	0
		F(2)	Suprasellar region						
CPG(5)	18(11-59)	M(3)	Sellar region,	0	0	0	0	0	5(100%)
		F(2)	Suprasellar region						

### Routine histology and immunohistochemical study of BRAF V600E mutation

Formalin fixed, paraffin-embedded (FFPE) tissue was sectioned at 4 µm and mounted on precoated glass slides(Thermo scientificsuperfrost plus, USA). Immunostaining for BRAF VE1 (clone VE1, Immunologic, Roche, USA) using the Opti ViewDAB kit (Immunologic, Roche, USA) was performed on a Ventana Bench Mark XT immunostainer (Ventana Medical Systems, Tucson, AZ, USA) as previously reported. BRAF V600E expression patterns were divided into four groups: 1) diffuse positivity referred to granular diffuse positivity in tumor cytoplasm; 2) reliable positivity of single tumor cells refers to the granular and definite positivity within the plasma in single tumor cells; 3) uniform "coating" positivity meant cytoplasmic positivity and varying degrees of nuclear positivity in tumor cell with a heavy background; 4) Negative meant that there is no expression in tumor cells with clear background. Immunohistochemical staining of GFAP, Olig-2, IDH-1, Syn, CgA, CD34, EMA, P53, ATRX, β-catenin and Ki-67 were also performed in some cases to aid diagnosis.

## Detection of BRAF V600E mutation by real-time qPCR

DNA samples were successfully obtained from a total of 120 tissue samples. PCR primers use in the present study were BRAF f 5'-TCATAATGCTTGCTCTGATAGGA-3'and BRAF r5'-GGCCAAAATTTAATCAGTGGA-3'. DNA was extracted from FFPE tissue using the FFPE extraction kit (Takara Bio, Japan) according to manufacturer's instructions. PCR settings were modified as follows: activation at 94°C for 10 mins, initial denaturation at 98°C for 10 s, annealing at 55°C for 30 s, extension at 72°C (1min/kb)for 35 cycles. DNA samples after purification were further quantified using Roche Cobas480 (Roche, USA).

## Result Analysis and Statistical Methods

The Image-Pro Plus 6.0 image processing and analysis software were used to measure the area, integrating absorbance(IA) of staining cells in every sample. They were divided into four groups according to immunohistochemical expression patterns, and the average IA of stained area (IA/area) was compared among the groups. The accuracy of immunohistochemical detection was verified by real-time qPCR. The BRAFV600E mutation prediction model was further established using Bayesian classification with tumor type, area of positive and average IA of stained area. All statistical analyses were performed in R (version R 3.6.3)

## Results

1. Among 120 cases of brain tumors, The Immunoreactivity Patterns of BRAF VE1 was Diffuse positivity (DP) in 55 cases, Reliable positivity of single tumor cell (RP-s) in 10 cases, uniform "coating" positivity (u-CP) in 20 cases, and negative in the other 35 cases. In the above four groups of cases with different expression patterns, the mean of average IA of stained area (IA/area) was  $0.134 \pm 0.067$ (n = 55) in DP group; was  $0.18 \pm 0.091$ (n = 10) in RP-s group; was  $0.046 \pm 0.014$ (n = 20) in u-CP group; and was  $0.018 \pm 0.022$ (n = 35) in negative group. There was no difference between DP group and RP-s group ( $P = 0.29$ ) in the average IA of stained area, while a significant difference in stained area ( $P = 0.000003$ ). Nevertheless, in the average IA of stained area, there was a significant difference between u-CP group and DP group( $P = 0.000002$ ), a difference between u-CP group and negative group( $P = 0,029$ ). (Table 2 and Fig. 1

Table 2

Analysis data of Image-Pro Plus 6.0 and molecular findings in various Immunoreactivity Patterns of BRAF VE1 staining

Groups(n)	Stained area(mean ± sd)	IA/area (mean ± sd)	BRAF V600E mutation(n)	BRAF wildtype(n)	P. Value of compare with DP in stained area	P. Value of compare with DP in IA/area	P. Value of compare with u-CP in IA/area
DP (55)	283915.9 ± 177435.7	0.134 ± 0.067	55	0	/	/	0.000002
RP-s (10)	4627.9 ± 3104.7	0.182 ± 0.0905	10	0	0.000003	0.28867	0.00003
u-CP (20)	162157.5 ± 130790.6	0.0461 ± 0.0152	2	18	0.05679	0.000002	/
N (35)	26401.5 ± 45758.2	0.0182 ± 0.0223	0	35	< 0.000001	<0.000001	0.029

2. The DP and RP-s groups were assumed to be positive for immunohistochemical interpretation, while u-CP and negative groups were assumed to be negative for immunohistochemical interpretation. The sensitivity of immunohistochemical detection was 97.01% and the specificity was 100%( $P < 0.0001$ ), which was detected by real-time qPCR.

3. The Immunoreactivity Patterns of BRAF V600E was different among different tumor types. The positive expression pattern of RP-s only presented in GC/GG, and p-CPG typically showed weak positive. The average IA of stained area of p-CPG was ( $0.088 \pm 0.019$ ), which was lower than other types of brain tumors( $P < 0.05$ ) (Table 3 and Fig. 2)

Table 3  
The Immunoreactivity Patterns of BRAF V600E in various brain tumor with BRAF V600E mutation

types(n)	Area(mean $\pm$ sd)	IA/area (mean $\pm$ sd)	P. Value of compare with p-CPG in IA/area
p-CPG(14)	113919.6 $\pm$ 77175.5	0.0876 $\pm$ 0.0190	/
DNT(2)	319268.5 $\pm$ 7675.6	0.223815 $\pm$ 0.023411	0.001
e-GBM(7)	289047 $\pm$ 209329.7	0.116366 $\pm$ 0.031983	0.05
GG(36)	256683.9 $\pm$ 213429.4	0.1611190.083677	0.00007
PA(1)	309580	0.12111	/
PXA(7)	325941.9 $\pm$ 136478.7	0.1312860.054764	0.001

Sixty cases were randomly selected from a variety of brain tumor types, a BRAF V600E mutation prediction model was established using Bayesian classification with tumor type, positive area and average IA of stained area. Results of the remaining 60 cases were used to verify the accuracy of the model, and the AUC value was 0.9525. (Fig. 3).

## Discussion

BRAF, a serine/threonine protein kinase belonging to the RAF family, is a key intermediary in the RAS-RAF-MEK-ERK-MAP kinase signaling pathway. It is also a key regulator of cellular functions, including cell proliferation, cell-cycle arrest, terminal differentiation, and apoptosis<sup>[11]</sup>. BRAF V600E mutation have been detected in several types of cancer, including a variety of tumors in central nervous system. The detection of BRAF V600E is essential in the diagnosis of brain tumors for the following reasons: 1) Some circumscribed gliomas (GGs and PXAs) are difficult to be differentiated from diffuse gliomas via histopathology alone, especially on biopsy specimens as they have different molecular characteristics. Therefore, BRAF-V600E detection has important differential diagnostic value<sup>[5]</sup>; 2) Detection of BRAF-V600E has a certain stratification effect on the prognosis of adult invasive glioma<sup>[12]</sup>; 3) With the clinical development of BRAF-targeted therapeutics, the detection of BRAF-V600E has important diagnostic and prognostic value<sup>[13]</sup>. However, the current molecular biology techniques are expensive and not yet widely available. If the BRAF-V600E expression status can be clarified through immunohistochemistry, it will be more efficient in the diagnosis and treatment evaluation for brain tumors. In melanoma, papillary cholangiocarcinoma, thyroid cancer, and colorectal cancer, VE1 detection has the specificity and sensitivity of more than 95%<sup>[2; 8; 9]</sup>, but few study has evaluated the sensitivity and specificity of BRAF VE1 immunochemical staining in central nervous system tumors. This study aimed to elucidate the current bias in the histopathological interpretation of BRAF VE1 staining, and provide the clinical insight into the potential future use of BRAF VE1 immunochemical staining in the diagnosis of brain tumor.

The gold standard for BRAF mutation analysis is direct sequencing of tumor DNA, but real-time qPCR tests were considered more effective and widely accepted in recent year<sup>[14]</sup>. In this study, the reliability of BRAF VE1 immunohistochemical staining was verified by real-time qPCR on the routinely processed formalin-fixed, and paraffin-embedded (FFPE) tumor tissue.

Our results suggested that all of the immunoreactivity patterns of diffuse positive cases for real-time qPCR detection were further confirmed to have BRAF V600E mutation (55/55,100%). None of the VE1-negative cases carried a BRAF V600E mutation (35/35,100%). It is important to note that reliable positivity within the plasma in single tumor cells generally suggested BRAF V600E mutations. What is more interesting is that the pattern of reliable positive of single tumor cell this single cell positive pattern

predominantly occurred in neuronal tumor cells of GG. It is similar as previously reported<sup>[15]</sup>. Circumspection was required for interpreting the immunohistochemical staining of BRAF VE1, especially for GC/GG. Our study indicated the pattern of completely negative IHC appeared to preclude the need mutational testing for BRAFV600E. And diffuse strongly positive cases in our experience equate with the presence of BRAF V600E mutation, reliable positive of single tumor cell in GG preferentially harbored BRAFV600E mutations, which might be exempt from molecular test for BRAF V600E in our practice.

In addition, uniform "coating" positivity with a heavy background occurred in some cases despite every attempt at optimization of the protocol. Our experience paralleled those reported by others<sup>[16; 17]</sup>. And uniform "coating" positivity was different from reliable diffuse positivity under a microscope, which was weak-moderate intensity positive in cytoplasmic and varying degrees of nuclear expression cell with a heavy background. In our study, the Image-Pro Plus 6.0 was used to measure the area, IA of staining cells in every sample. And the results showed that there were Significant differences in IA/area between DP groups and u-CP groups( $P = 0.000002$ ), also between DP and negative groups( $P < 0.000001$ ). Although there was a difference between u-CP group and negative group( $P = 0.029$ ), we prefer to interpret them as equivocal positivity. Similar to previous reports<sup>[16; 17]</sup>, the u-CP group is more likely to be interpreted without BRAF V600E mutation in our study (18/20,90%). Given the importance of accurate documentation of the BRAF mutational status prior to potential targeted therapies<sup>[18; 19]</sup>, we propose to continue molecular testing to further determine the BRAF gene status in these ambiguous cases.

What's the reason that the u-CP immunoreactivity pattern of BRAF VE1 fail to interpret as gene mutation was unknow. Some argued that this is mainly due to the limitations of commercial antibodies<sup>[16]</sup>, while others<sup>[20]</sup> speculated that the epitope of BRAF VE1 antibody shared structural homolog to the epitope of completely unrelated molecules in some protein-rich cells, such as ganglionic-like or ganglion cells<sup>[20]</sup> and pituitary adenoma cells<sup>[21]</sup>. Therefore, while staining of BRAF VE1 protein may appear as a feasible method for evaluating BRAF mutation status, it is important to confirm the presence of BRAFV600E mutation by molecular test when the Immunoreactivity Patterns is u-CP, especially in some types of tumors not known to, or rarely harbor such mutations.

It was very interesting that p-CPGs typically showed weak to moderate staining for BRAF VE1, while always harbored BRAF V600E mutations. On the contrary, adamantinomatous craniopharyngioma was always negative for VE1, while aberrant nuclear accumulation of beta-catenin could be detected by immunohistochemistry<sup>[22]</sup>. Therefore, we can distinguish p-CPG from adamantinomatous craniopharyngioma by BRAF VE1 staining. When the craniopharyngioma is associated with cystic degeneration due to the cyst wall epithelium atrophies caused by the pressure of the cyst, it is difficult to differentiate from Rathke cleft cysts in sellar region with epithelial squamous metaplasia, which can be resolved by conducting immunohistochemistry. Unlike craniopharyngioma, Rathke cleft cysts are known for the lack of BRAF V600E mutations and cell membrane accumulation of beta-catenin localizes<sup>[23]</sup>. In addition, BRAF-V600E status can provide an important evidence for the targeted therapies of recurrent and refractory craniopharyngioma<sup>[24]</sup>.

In summary, we found that BRAF VE1 staining could replace molecular detection to some extent, on the premise of mastering the key points in the interpretation of BRAF VE1 immunostaining. Key points of interpretation including: 1) as long as the positive signal was accurately located in the cytoplasm of tumor cells, the sample was considered to have BRAF V600E mutation, disregarding the number of staining cells, especially for GG; 2) for craniopharyngioma, even a weak positive for BRAF VE1 staining, a diagnosis of papillary craniopharyngioma harbored BRAF V600E mutation should be considered. 3) Tissue samples that had no signal of BRAF VE1 expression with clear background could be confirmed without BRAF-V600E mutation. 4) Some equivocal positive with uniform "coating" positive cases, which were often considered as false-positive and usually required further molecular detection. Otherwise, the models for predicting BRAF mutations of BRAF VE1 by tumor type, positive area and average IA of stained area may provide a possible scheme for artificial intelligence interpreting of immunohistochemical staining.

## Declarations

### FUNDING

This study was funded by the Guangdong Medical Science and Technology Research Foundation (No. A2019315; B2021250).

### CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

**AUTHORS' CONTRIBUTIONS** Hai Li, Chongzu Fan and Minting Liu analyzed the data, performed the experiments and wrote the main manuscript text. Wendan Chen, Baijie Chen, Xuan Xiang, Zhenbin Zhang performed some of the experiments and provided essential material. Linbo Cai and Zhi Li supervised the study and critically reviewed the manuscript. All authors reviewed the manuscript.

**DATA AVAILABILITY** Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

**ETHICS APPROVAL** The study was approved by the Ethics Committee (EC number: 2020-010-087) of Guangdong Sanjiu Brain Hospital and was conducted according to the Declaration of Helsinki.

### Consent to participate

Informed consent was obtained from all participants.

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## Figures

### Figure 1

#### **H&E staining and BRAF VE1 Immunoreactivity Patterns in various brain tumors.**

1a: Histopathologic features of cases diagnosed as e-GBM. 1b-1f: Diffuse positive expression patterns in various brain tumors, 1f showed weak positive in p-CPG; 1g-1l: showed histopathologic features of cases diagnosed as GG, and reliable positivity of single tumor cells in GC/GG; 1m: showed morphological diagnosed as PXA; 1n-1r: showed uniform "coating" positivity patterns of cytoplasmic positivity and varying degrees of nuclear positivity in tumor cell with a heavy background; 1s: presented morphological diagnosed as p-CPG; 1t-1x: presented negative patterns with clear background.

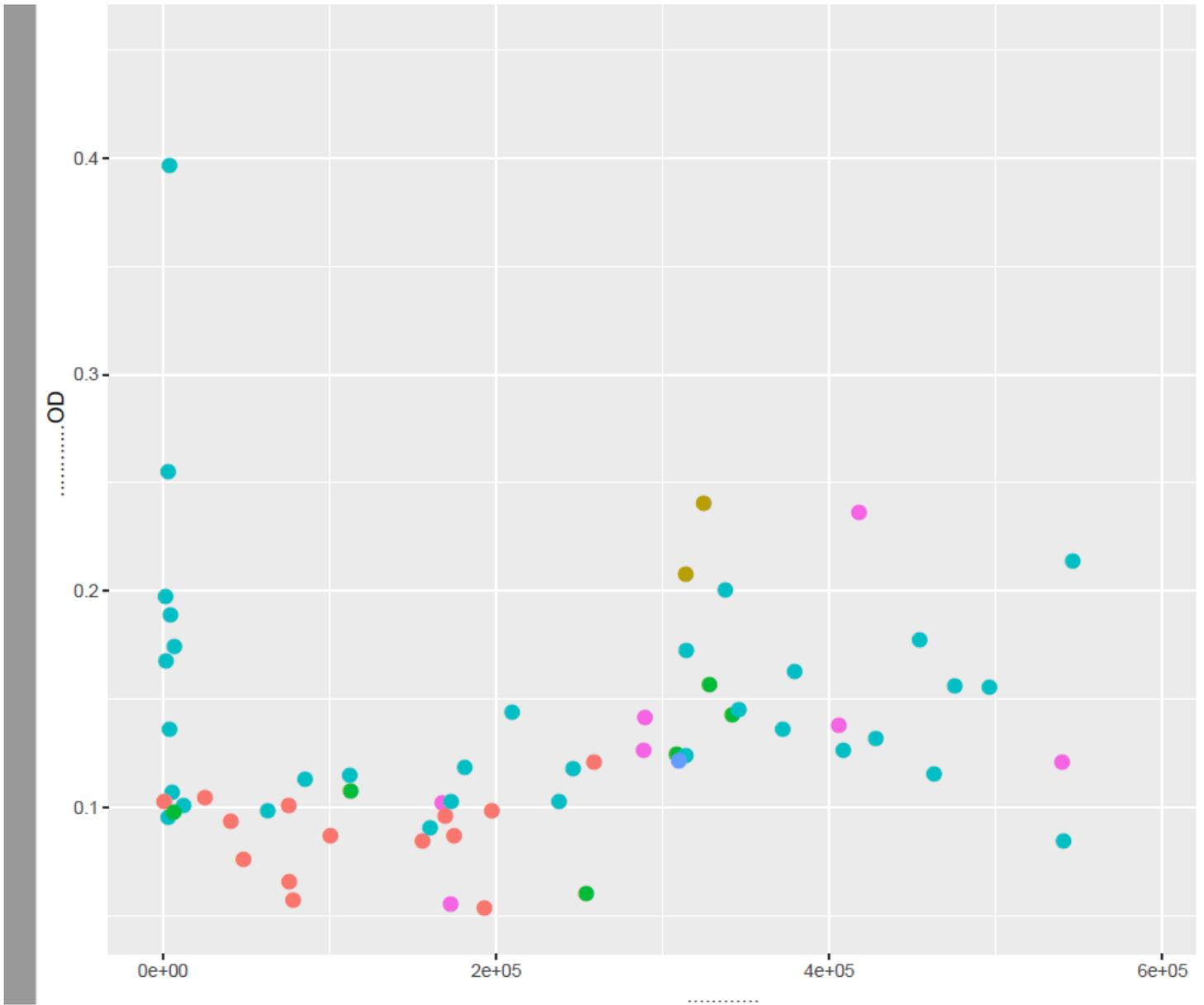


Figure 2

Stained area and the average IA of stained area in various brain tumors harbored BRAF V600E mutation. Expression pattern with low stained area always presented in GC/GG, the positive pattern of p-CPG frequently had a low average IA of stained area.

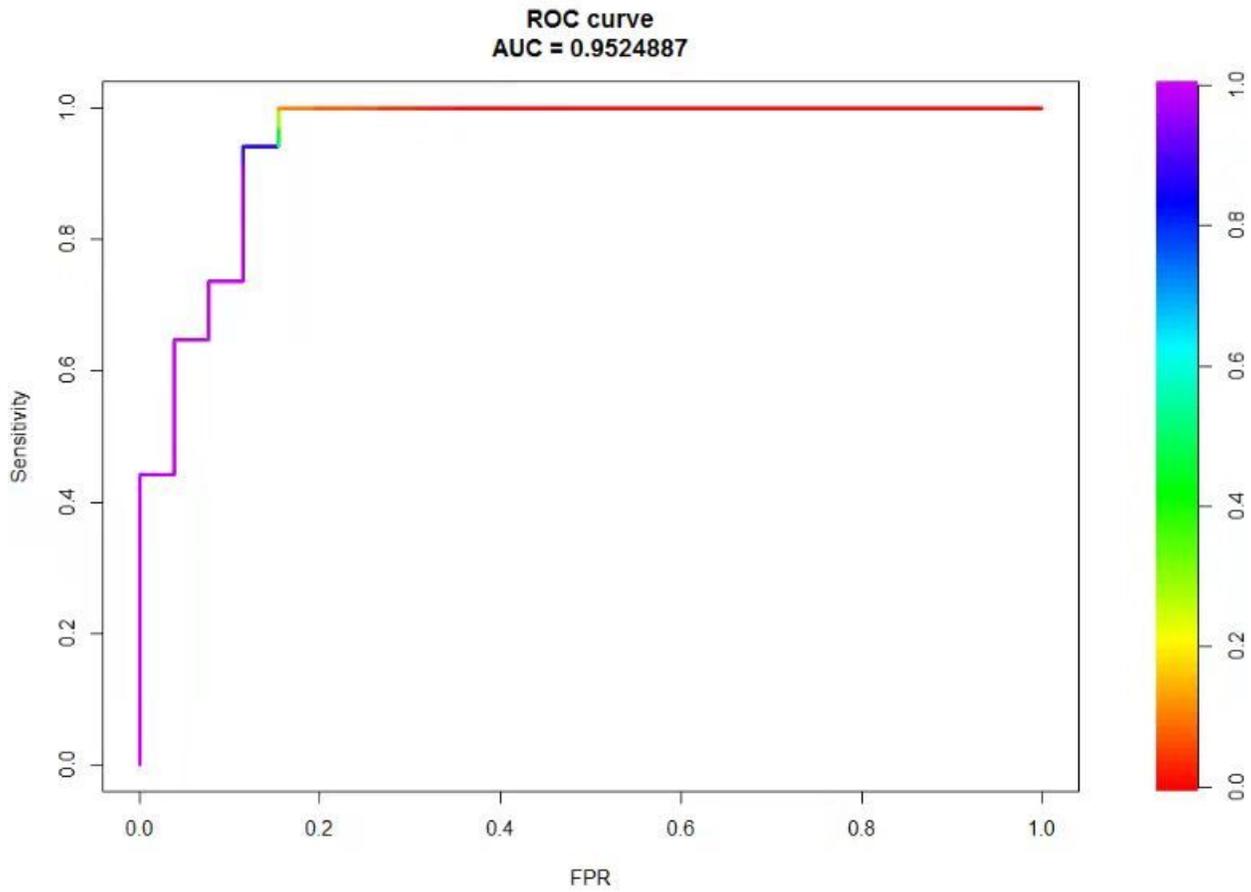


Figure 3

**The prediction model of Bayesian classification for BRAF VE1**

BRAF V600E mutation was predicted based on stained area and average IA of stained area of BRAF VE1 staining combined with tumor type, with an AUC value of 0.9525.