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Skin Cancer

BRAF V600E Mutations and Beyond: A Molecular Perspective of Melanoma from a Tertiary Cancer Referral Center of India

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Abstract

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Objectives Malignant melanoma demonstrates frequently occurring mutations of genes in the serine/threonine kinase pathway, namely BRAF, NRAS, and neurofibromin 1. There is rare documentation of a detailed analysis of these mutations in cases of melanoma among Indian patients. We present molecular features in cases of malignant melanoma, diagnosed at a tertiary cancer referral center in India, over a period of 8 years (2011–2018).

Materials and Methods This study was performed on formalin fixed paraffin embedded tissues of 88 histologically confirmed cases of malignant melanoma. BRAF gene alterations were studied by both Sanger sequencing and real-time polymerase chain reaction techniques ($n = 74$). Molecular testing for BRAF and NRAS gene alterations was accomplished in 74/88 cases (80%). Molecular test results were correlated with clinicopathological features using IBM SPSS Statistical software 25.0.

Results The age ranged from 13 to 79 years (median = 57), with a M:F ratio of 1.4:1. BRAF mutations were observed in 12/74 (16.21%) patients, including V600E ($n = 7$), A594T ($n = 1$), T599 = ($n = 2$), V600K ($n = 1$), and Q612P ($n = 1$), while NRAS mutations were observed in 6/38 (15.7%) patients. Among various subtypes, nodular melanoma was the most frequent subtype (33%) among cutaneous malignant melanomas. Among non-cutaneous melanomas, mucosal melanomas were observed in 37.5% of cases.

Conclusion This constitutes one of the few reports on comprehensive analysis of molecular alterations underlying melanomas in Indian patients. A larger sample size, with more extensive molecular markers, would yield additional information on the disease manifestation.

Keywords
- Indian population
- BRAF V600E mutations
- melanoma
- molecular alterations
- NRAS mutations

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Introduction

Malignant melanoma (MM) is a rare type of skin cancer that develops from the melanin-producing melanocytic cells and is responsible for most cutaneous cancer-related deaths, whereas noncutaneous melanomas comprising of ocular and various mucosal sites such as anorectal, vaginal, nasal, and gastrointestinal tract are very rare.[1]

MM is considered as one of the most highly mutated, heterogeneous, and lethal types of cancer with an average of 16.8 mutations per Mb according to The Cancer Genome Atlas data.[2] The most frequently identified mutations are the serine/threonine kinase *BRAF* (50%), the small GTPase *NRAS* (25%), followed by the tumor suppressor and negative regulator of *RAS*, neurofibromin 1 (*NF1*) (14%).[3,4]

These mutations often cause upregulation of the mitogen-activated protein kinase (MAPK) pathway, leading to increased proliferation and survival of tumor cells.[3] Environmental factors play a major role in genetic alterations. Patients with a history of intermittent sun exposure are more prone to harbor *BRAF* mutations than the ones who are chronically exposed.[5]

Previous studies investigating the role of *BRAF* and *NRAS* as independent prognostic markers have shown discordant data.[6,7] A few studies showed that patients harboring *BRAF* V600E mutations had a relatively lower overall survival (OS) and disease-free survival (DFS), as compared to those harboring the wildtype *BRAF* gene, while other studies showed that the presence or absence of *BRAF* V600E mutation failed to influence the OS.[8,9] A relatively higher *BRAF* expression has also been found to be related to tumor ulceration and metastasis, in some studies.[10,11] Likewise, some studies have shown *NRAS* as an independent prognostic marker, while others have not shown a correlation between *NRAS* gene alteration and OS.[7,12] Until recently, the treatment options for advanced stage melanoma patients were limited to conventional chemotherapeutic drugs with an overall low efficacy and limited response rate. Only in the past few years, the progression-free and OS of melanoma patients have markedly improved by the introduction of targeted therapy and immunotherapy.[3]

According to the GLOBOCAN 2020 statistical data, the incidence of MM is comparatively lower in Asia (7.3%) as compared to North America (32.4%) or Europe (46.4%).[13] As per the Tata Memorial Hospital registry, MM constitutes 0.3% of the total cases reported.[14] Interestingly, some studies have shown a distinct prevalent histopathological subtype; different sites of presentation, risk factors, as well as underlying mutations, in cases of MM occurring within Asian patients.[15]

Currently, there is sparse literature describing the mutation spectrum in MM among the Indian population. Considering the ethnic, geographical, and regional variation across the Indian subcontinent, the MM cases presented from this country would probably have a diverse presentation ranging from histology to cell type as well as the underlying mutations.[16–20] Herein, we present molecular alterations in cases of melanoma diagnosed at a tertiary cancer referral center in India over a period of 8 years (2011–2018).

Materials and Methods

Tumor Samples

The study included an analysis of 88 consecutive cases of melanoma diagnosed in the Department of Pathology of our Institution, from January 2011 to December 2018 (8-year duration). Hematoxylin and eosin stained slides of all the cases were reviewed, especially to determine tumor adequacy.

Molecular analysis was conducted on representative formalin-fixed paraffin-embedded (FFPE) tissues. Clinical and demographic details were collected from the electronic medical record of the Institution.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on an automated immunostainer (Benchmark XT, Ventana Medical Systems Inc., Arizona, United States). Details of the various antibodies, including S100P, HMB45, Melan A, and AE1/AE3, are listed in ►**Supplementary Table S1** (available in the online version).

BRAF gene alterations were studied by both Sanger sequencing ($n = 74$) and real-time polymerase chain reaction (RT-PCR) methods ($n = 74$), to confirm the results.

DNA Extraction

After confirmation of tumor content and adequacy, the selected FFPE blocks were subjected to genomic DNA extraction from four sections each of 10µm thickness. Sections were deparaffinized using limonene (Sigma Aldrich, Missouri, United States) followed by overnight digestion and DNA extraction using the QIAamp DNA Mini Kit (Cat. 56404; Qiagen, Hilden, Germany) as per manufacturer's instructions. Extracted DNA was checked for quality (260:280 ratio) and quantity by NanoDrop 2000 (Thermo Fisher Scientific, Massachusetts, United States). The integrity of the DNA was assessed by PCR for the β-actin (*ACTB*-208bp) housekeeping gene (►**Supplementary Table S2**, available in the online version) and the samples showing *ACTB* amplification were selected for molecular analysis.

Molecular testing could be accomplished in 74/88 (80%) cases. In the remaining 14 cases, molecular testing was not possible, either due to suboptimal quality of the DNA or uninterpretable sequencing data.

BRAF, *NRAS* (Exon 2 and Exon 3) PCR and Sequencing

Briefly, PCR amplification was carried out using 2X PCR master mix (Thermo Fisher Scientific, Massachusetts, United States), 1 µL each of 10pmol forward and reverse primer (►**Supplementary Table S2**, available in the online version) and 100 ng of template DNA. PCR was carried out as per conditions mentioned in ►**Supplementary Table S3** (available in the online version). Direct DNA sequencing was performed on the purified PCR products with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Massachusetts, United States) followed by purification using the Optima DTR plates (Edge BioSystems, California, United States). Sequencing was conducted on the ABI 3500 Genetic Analyzer (Applied Biosystems, Massachusetts, United States).

Sequence Analysis

Sequences were analyzed using the Chromas Lite version 2.0 software and compared with the reference sequence of *BRAF* (Gene ID:673) and *NRAS* (Gene ID:4893) genes. Mutations were reported as per the Human Genome Variation Society (www.hgvs.org) recommendations.

RT-PCR Assay for Detecting *BRAF* V600 Mutation

The RT-PCR assay was used to detect *BRAF* V600E mutations using the TaqMan Gene Expression master mix on the Quant Studio 12K Flex System (Thermo Fisher Scientific, Massachusetts, United States). The sequence for the wildtype *BRAF* and *BRAF* V600E probes was designed as per literature[21] as mentioned in ►**Supplementary Table S2** (available in the online version). The assay was set up in triplicates for both the genotypes as per cycling conditions mentioned in ►**Supplementary Table S4** (available in the online version). Results were analyzed on the Quant Studio expression suite software (Thermo Fisher Scientific, Massachusetts, United States).

Statistical Analysis

Molecular results were correlated with the clinicopathological features, including age of the patient, tumor site, histopathological subtype, and geographical location, using IBM SPSS Statistical software 25.0. The data was summarized using descriptive statistics. Data pertaining to continuous variables, such as age, were described using the mean ± standard deviation of the median (range) for normally distributed data. Pearson's chi-squared test was used to differentiate the rates between the different groups.

Results

Clinicopathologic Features

The study comprised of patients across four regions of the country, predominantly from the west (39/88; 44.3%); followed by from the east (29/88; 33%), north (15/88; 17%), and the southern (5/88; 5.6%) region, respectively.

Eighty-eight cases of MM occurred in patients, with age ranging from 13 to 79 years (median = 57); in 51 males and 37 females, with a M: F ratio of 1.4:1. Among the 88 cases studied, 10 were referral cases (►**Table 1**).

The predominant sites involved were skin and soft tissues: the gastrointestinal tract and the genitourinary tract. The most common histopathologic subtypes of cutaneous MM ($n = 47$) were nodular melanoma (NM) (29/88; 33%), followed by acral lentiginous melanoma (ALM) (14/88; 15.9%), superficial spreading melanoma (SSM) (2/88; 2.3%), and desmoplastic melanoma (2/88; 2.3%). Among noncutaneous MMs ($n = 37$), there were cases of conjunctival MM (CMM) (4/88; 4.5%) and mucosal melanoma (33/88; 37.5%). There were four cases of metastatic melanoma with unknown primary sites ($n = 4$; 4.5%). Details regarding Clark's level of invasion were available in 22 cases, among which predominant cases were between stages III to V, including 10 patients (45%) with stage V disease (►**Table 1**).

IHC results were available in 56 cases (63.6%). The various IHC markers studied are listed in ►**Table 2**.

Table 1 Demographic and clinical characteristics of the study cohort

Characteristics		No. of cases
Gender	Male	51 (58.0%)
	Female	37 (42.0%)
Age	>65	17 (19.3%)
	<65	71 (80.7%)
Histological classification	Superficial spreading melanoma (SSM)	2 (2.3%)
	Acral lentiginous melanoma (ALM)	14 (15.9%)
	Nodular melanoma (NM)	29 (33%)
	Desmoplastic melanoma (DM)	2 (2.3%)
	Conjunctival melanoma (CM)	4 (4.5%)
	Mucosal melanoma (MM)	33 (37.5%)
	MM of unknown primary (MUP)	4 (4.5%)
Clarks level	II	0
	III	4 (18.2%)
	IV	8 (36.4%)
	V	10 (45.5%)
Geographical location	East	29 (33.0%)
	West	39 (44.3%)
	North	15 (17.0%)
	South	5 (5.7%)

Molecular Results

BRAF Gene Alteration

Among the 88 study cases, *BRAF* gene sequencing was performed in 74 cases.

BRAF mutations were observed in 12/74 (16.21%) tested cases. Both *BRAF* V600E and non-V600E mutations were observed, including V600E ($n = 7$), A594T ($n = 2$), T599 ($n = 2$), V600K ($n = 1$), and Q612P ($n = 1$). This included a single case, displaying dual *BRAF* mutations (V600E and non-V600E).

Of the 12 patients harboring *BRAF* mutations, 9 (75%) were males. Based on the histopathologic subtypes, *BRAF* mutations were observed in 8.3% cases of SSM ($n = 1/12$), 16% cases of ALM ($n = 2/12$), 25% cases of NM ($n = 3/12$), 8.3% cases of CMM ($n = 1/12$), 33.3% cases of mucosal melanoma ($n = 4/12$), and in 8.3% cases of MM with unknown primary site ($n = 1/12$).

Immunohistochemically, all 8/12 cases of *BRAF*-mutant melanomas were positive for HMB45; 8/12 were positive for S100P and 6/12 cases were positive for Melan A, wherever these markers were tested (►**Table 2**).

A 73-year-old male patient harboring ALM, with distant metastasis (lung, nodes, brain), revealed the presence of dual mutations, namely Q612P and A594T of the *BRAF*

Table 2 Correlation of *BRAF* and *NRAS* alterations with the clinical features

Characteristics			*BRAF* status			*NRAS* status		
			Total (*n*)	Mutant n (%)	Wildtype *n* (%)	Total (*n*)	Mutant *n* (%)	Wildtype *n* (%)
			74	12 (16.21%)	62 (83.7%)	38	6 (15.8)	32 (84.2)
Sex	Male		42	9 (21.4%)	33 (78.5%)	25	3 (12%)	22 (88%)
	Female		32	3 (9%)	29 (90%)	13	3 (23%)	10 (77%)
Tumor type	SSM		2	1 (50%)	1 (50%)	1	1 (100%)	0
	ALM		13	2 (15.4%)	11 (84.6%)	7	2 (28.6%)	5 (71.4%)
	Nodular M		24	3 (12.5%)	21 (87.5%)	11	0	11 (100%)
	Conjunctival M		3	1 (33.3%)	2 (66.6%)	0	0	0
	Mucosal M.		27	4 (14.8%)	23 (85.2%)	18	2 (11.1)	16 (88.8%)
	Desmoplastic M.		1	0	1 (100%)	0	0	0
	MM with unknown primary		4	1 (25%)	3 (75%)	1	1	0
IHC markers	HMB45	Positive	40	8 (20%)	32 (80%)	22	5 (22.7%)	17 (77.2%)
		Negative	5	0	5 (100%)	2	0	2 (100%)
	Melan A	Positive	28	6 (21.4%)	22 (78.5%)	13	5 (38.4%)	7 (53.8%)
		Negative	5	0	5 (100%)	2	0	2 (100%)
	AE1/AE3	Positive	2	0	2 (100%)	2	0	2 (100%)
		Negative	14	1 (7.14%)	13 (92%)	7	1 (14.2%)	6 (85.7%)
	S100	Positive	43	8 (18.6%)	35 (81.3%)	23	4 (17.3%)	19 (82.6%)
		Negative	1	0	1 (100%)	0	0	0

Abbreviations: ALM, acral lentiginous melanoma; IHC, immunohistochemistry; MM, mucosal melanoma; SSM, superficial spreading melanoma.

gene. RT-PCR analysis was performed in all 74 cases. The concordance between RT-PCR assay and capillary electrophoresis was found to be 100%.

NRAS Gene Alteration

NRAS mutations were observed in 6/38 (15.7%) patients, including 3 males and 3 females. The various types of *NRAS* mutations observed were G13D ($n=2$), A59V ($n=1$), Q61R ($n=2$), and S17 ($n=1$). These mutations were observed in 33.6% cases of ALM ($n=2/6$), 33.3% cases of mucosal melanoma ($n=2/6$), 16.6% cases of SSM ($n=1/6$), and 16.6% cases of metastatic melanoma with an unknown primary site ($n=1/6$; ►Figs. 1–6; ►Table 2).

In two patients, both *BRAF* and *NRAS* gene mutations were observed, including a 52-year-old lady with metastatic MM in her right inguinal lymph node, harboring mutations in both *BRAF* (V600E) and *NRAS* (G13D) genes. Another patient was a 57-year-old male, with MM of the right inguinal lymph node, with unknown primary, harboring mutations in the *BRAF* (V600E) and *NRAS* (Exon2 G13D) genes. *NRAS* and *BRAF* gene alterations are mostly mutually exclusive. The coexistence of both *BRAF* and *NRAS* mutations could be due to clonal heterogeneity in the tumor.

The clinicopathological features of melanoma cases with *BRAF* and *NRAS* gene alterations are depicted in ►Table 3.

Regional Distribution of *BRAF* and *NRAS* Mutations

The incidence of *BRAF* and *NRAS* alterations based on the geographical location of the patients was analyzed. Fifty-eight percent of cases ($n=7/12$) with *BRAF* mutations were from the Eastern region, while 50% ($n=3/6$) cases with *NRAS* mutations belonged to the Western region. No cases harboring the *BRAF* and *NRAS* mutations were from the South and North India regions respectively (►Table 4).

Discussion

Globally, approximately 1,60,000 new cases of MM are diagnosed each year.[22] The worldwide incidence of melanoma continues to rise, with Australia having the highest incidence, followed by Europe and the United States. Molecular alterations, such as the *BRAF* V600E mutation, have been reported in nearly 40 to 45% of the cases in studies from these countries.[11,23,24]

An increased incidence of uveal melanoma has been reported in countries, including France, Italy, and Japan.[25,26] A relative higher frequency of mutation in the eastern region, closely followed by the west, is indicative of regional variation among the Indian cohort. India constitutes one of the relatively low incidence regions for MM in the world.[27] At Tata Memorial Hospital, which is the premier cancer referral center of India, the incidence of MM is 0.3% among cutaneous malignancies and even rarer among non-cutaneous malignancies.[14]

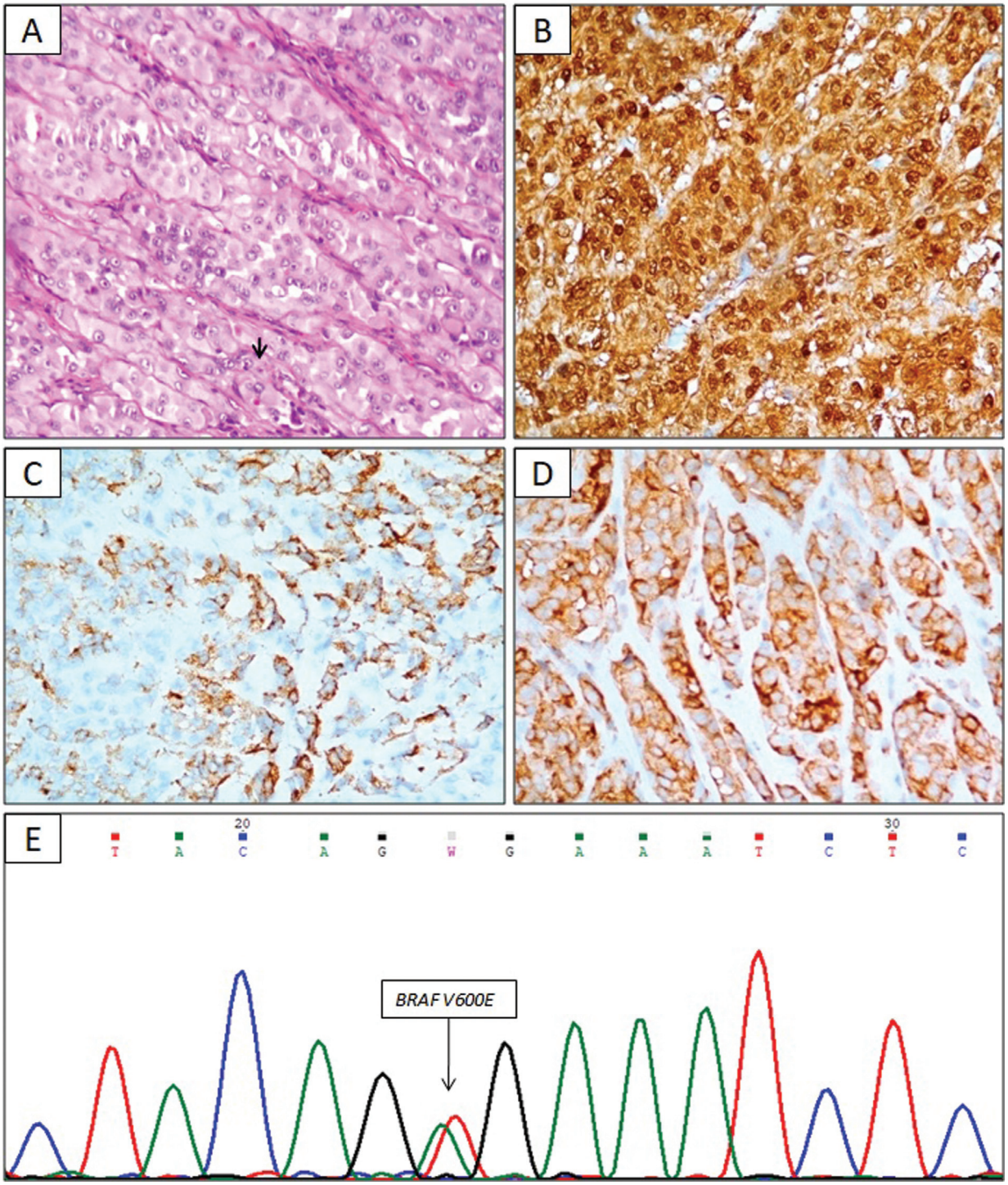

Fig. 1 Nodular melanoma, *BRAF* mutant (A–E): (**A**) Tumor composed of epithelioid cells arranged in a nested pattern with very focal melanin pigment (arrow). Hematoxylin and eosin x400. (**B**) Diffuse nuclear and cytoplasmic positivity for S100P. DAB x 400. (**C**, **D**) Positive staining for HMB45 and MelanA immunohistochemistry. DAB x 400. (**E**) Electropherogram showing *BRAF* Exon 15 p.Val640Glumutation.

This study describes the frequency of molecular alterations underlying melanomas in Indian patients at a tertiary cancer referral center. The frequency of *BRAF* mutations in this study was 16.21% and that of *NRAS* was 15.7%. While the incidence of *BRAF* mutations seems to be on the lower side of the reported range, which is 16 to 41%, across different studies; the frequency of *NRAS* mutations was higher than the range of 4 to 10%, reported in different studies.[28–30] In this study, the observed rates of *BRAF* mutations were less, as compared to those reported from Japan (41.4%), Russia (43.3%), and China (23%), in three different studies.[1,31,32] Among studies from various Asian countries (►**Table 5**), comparable incidence of *BRAF* mutation was observed in Taiwanese (14.3%) and Indonesian patients (10%). The rate of *NRAS* mutation in Taiwanese patients was 10.1%. In another study from Korea, the rates of *BRAF* (6.4%), as well as *NRAS*

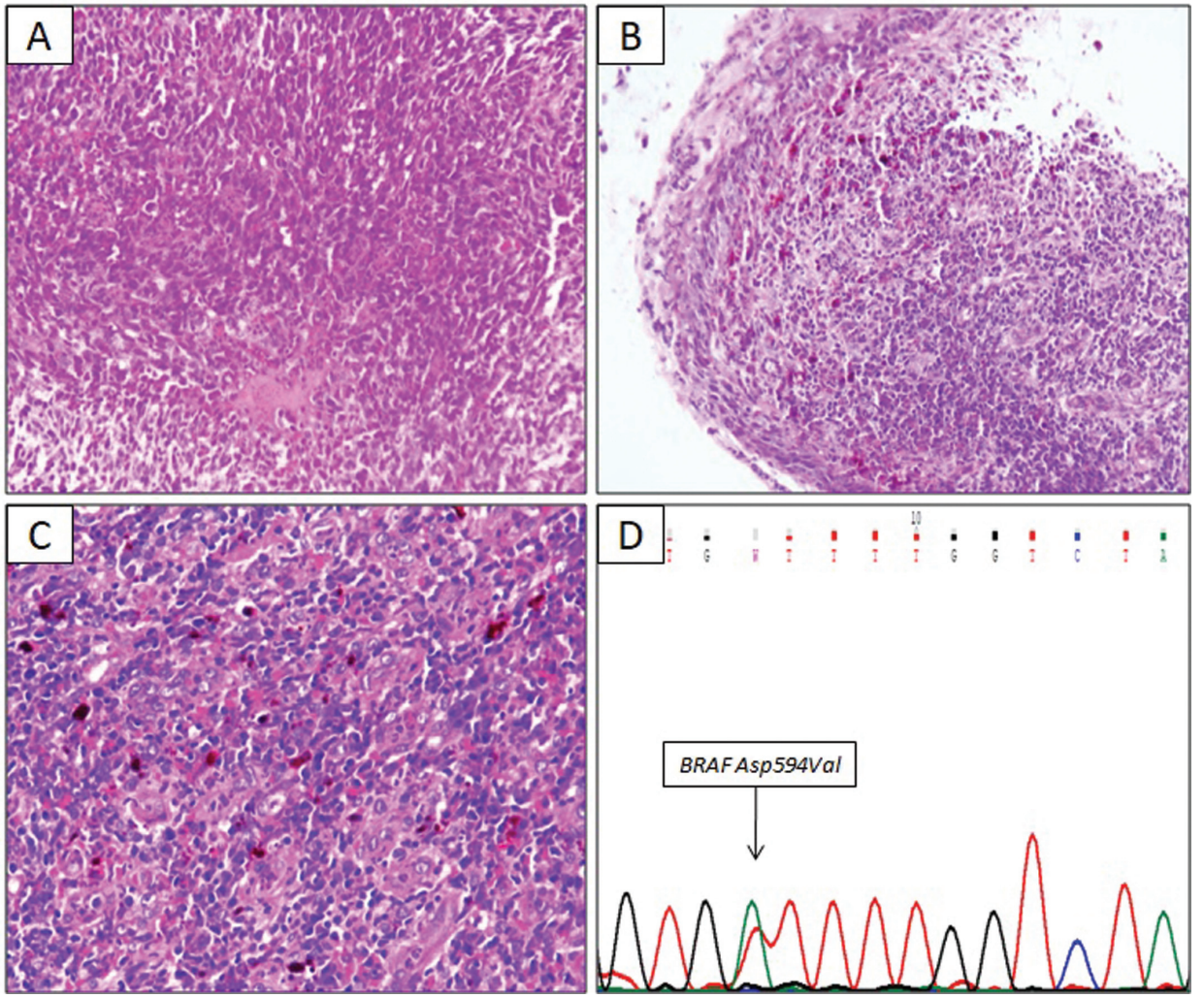

Fig. 2 Mucosal melanoma, *BRAF* mutant (A–D): (**A**) Biopsy from the anorectal mass shows tumor cells arranged in sheeted pattern. Hematoxylin and eosin (H&E) x100. (**B, C**) Melanin pigment noted H&E x100. (**C**) H&E x 200. (**D**) Electropherogram showing *BRAF* Exon 15 p. Asp594Val mutation.

mutations (4.3%) in such patients were lowest.[28–30] An earlier study from India revealed a 30% incidence of *BRAF* mutation in cases of MM.[19] On comparing the last 5-year published data on *BRAF* mutations among Asian patients with MM (►**Table 5**), all studies except that of Ahmad et al[19] utilized RT-PCR or Sanger sequencing, with an assay sensitivity and limit of detection of up to 10% tumor content. Hence, the difference in the frequency of mutation between this study (16.7%) and by Ahmad et al (30%)[19] could possibly be due to the choice of techniques, tumor content, sample selection criteria, as well as the assay sensitivity.

Among various subtypes of cutaneous melanoma in this study, NM ($n = 29$; 33%) was the most frequent subtype, while in noncutaneous melanomas, mucosal melanoma was commonly observed ($n = 33$; 37.5%). *BRAF* mutations were observed in mucosal melanomas ($n = 4/12$), as well as NM ($n = 3/12$), while *NRAS* mutations were predominantly observed in ALM ($n = 2/6$) and mucosal melanomas ($n = 2/6$). These observations were different compared to the previous studies.[28,33–35] A meta-analysis of 19 studies on the frequency of *BRAF* mutations across various subtypes of MM revealed that *BRAF* mutations are frequently associated with the SSM subtype.[36] In their study, Yamazaki et al[31] also reported an association of *BRAF* mutation with the SSM subtype. Considering a significant number of our patients present with advanced lesions, with nodular MM as the commonest subtype, a relatively higher percentage of *BRAF* mutations were noted in that subtype.

According to the World Health Organization report, ultraviolet (UV) radiation- related disease such as melanoma is predominant in the fair-skinned population belonging to the European, Western Pacific region, and the American region, while it is uncommon in the African, Eastern Mediterranean region and South East Asian regions.[37] Most cases of the melanoma diagnosed among Africans and Asians include the ones occurring in palms, soles, mucous membrane and subungual sites.[38] This observation is consistent with the findings in this study. Moreover, a significant number of studies from Asian countries have shown ALM as the most frequent subtype. Those studies have shown a relatively lower incidence of *BRAF* mutation in ALM, as compared to that in SSM.[39,40]

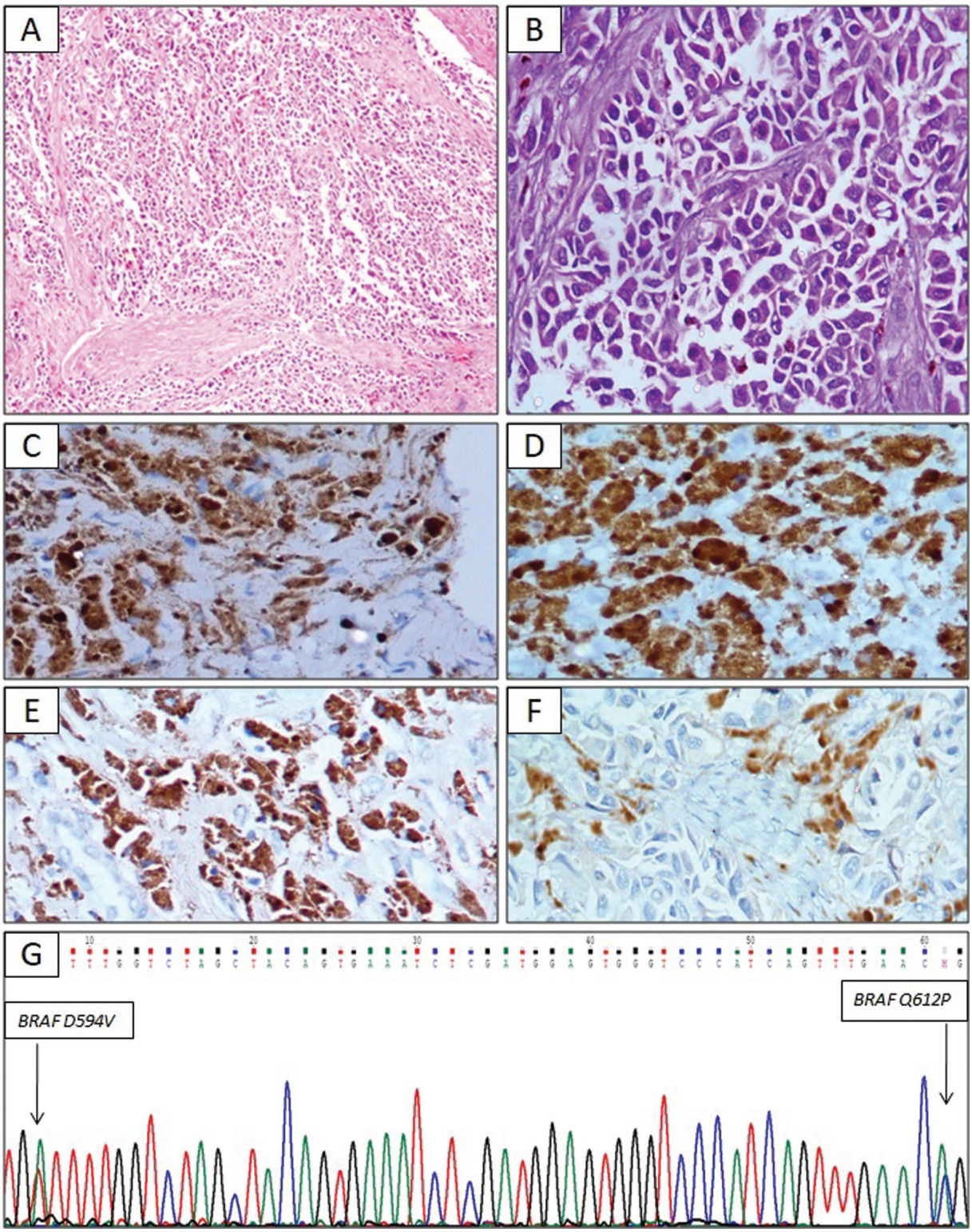

Fig. 3 Acral lentiginous melanoma, *BRAF* mutant (**A**–**G**): (**A**) Tumor arranged in nested and at places alveolar pattern with presence of melanin pigment. Hematoxylin and eosin (H&E) x 100. (**B**) Tumor cells have epithelioid morphology and show intracellular melanin pigment. H&E x400. (**C**) S100P shows diffuse nuclear and cytoplasmic positivity. DAB x400. (**D**, **E**) Cytoplasmic positivity for MelanA and HMB45 respectively. DAB x400. (**F**) Patchy positivity for EMA. DAB x400. (**G**) Electropherogram showing *BRAF* Exon 15 harboring dual mutation p.Asp594Val andp.Gln612Pro.

It has been observed that *BRAF* mutation does not occur at the initiation of tumorigenesis, but it is important in the progression of cutaneous melanoma. It is important to note that the difference in incidence of *BRAF* mutations in different subtypes of melanoma could be related to etiological factors, such as UV exposure.

The frequency of *NRAS* mutation in this study was slightly higher than in the other two reported studies from Asia,[28,29]

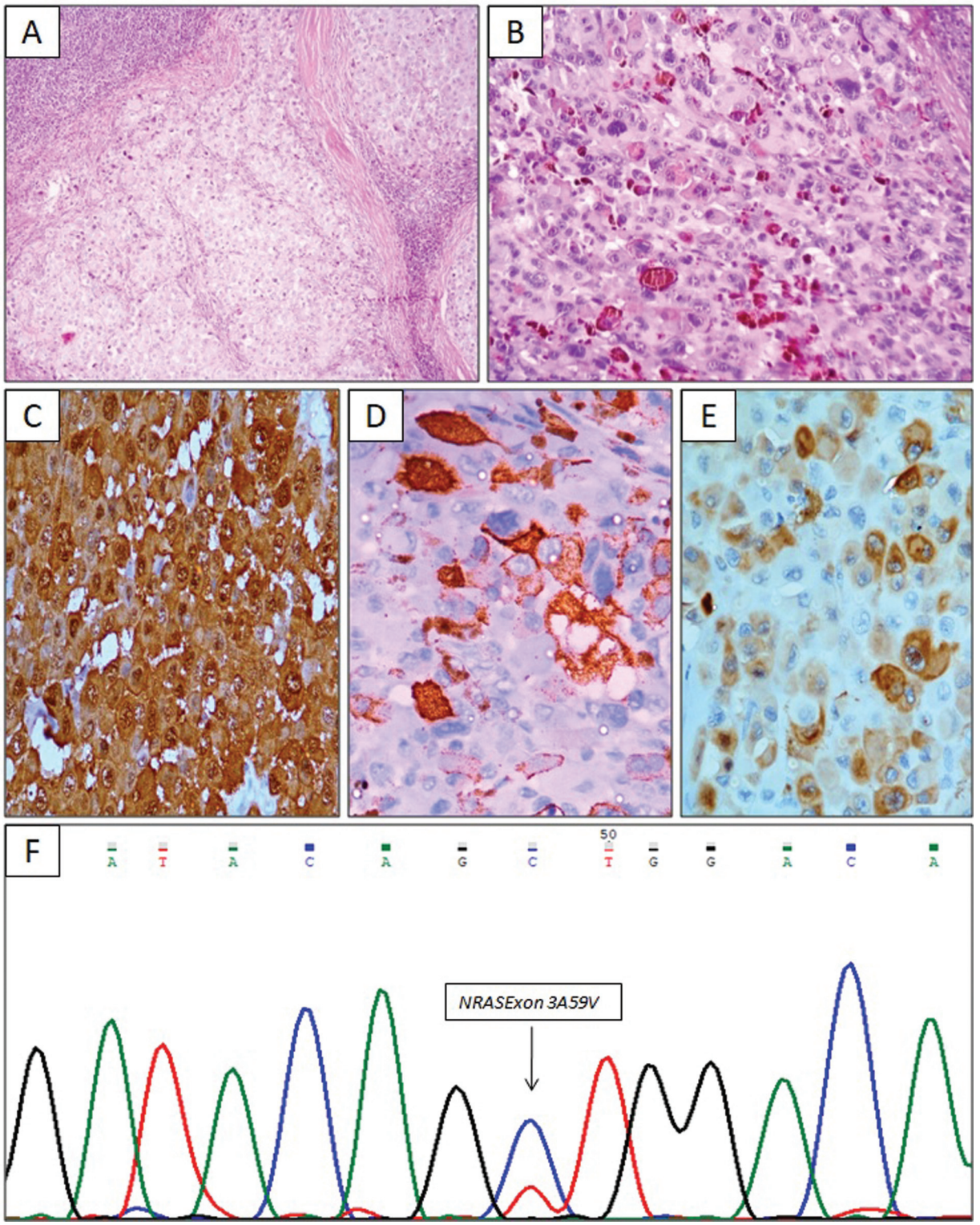

Fig. 4 Metastatic melanoma; unknown primary, *NRAS* mutant (A–F): (**A**) Tumor deposits in lymph node composed of nodules of epithelioid cells. Hematoxylin and eosin (H&E) x100. (**B**) Tumors cells showing intracellular melanin pigment and prominent nucleoli. H&E x200. (**C**) Diffuse nuclear as well as cytoplasmic positivity for S100P. DABx400. (**D**) Distinct cytoplasmic positivity for MelanA.DABx400. (**E**) Cytoplasmic positivity for HMB45. DABx 400. (**F**) Electropherogram showing *NRAS* Exon 3 Ala59Val mutation.

whereas it was slightly lower as reported in the Caucasian patients.[7,41]

Based on various geographical regions, we observed that the patients afflicted with MM from the eastern part of the country had the highest number of *BRAF* gene alterations (58.3%), while patients from the western part of the country had the maximum number of *NRAS* gene alterations (50%). Nodular and mucosal melanoma were the most frequently observed subtypes. However, this finding was not statistically significant because of the relatively limited sample size.

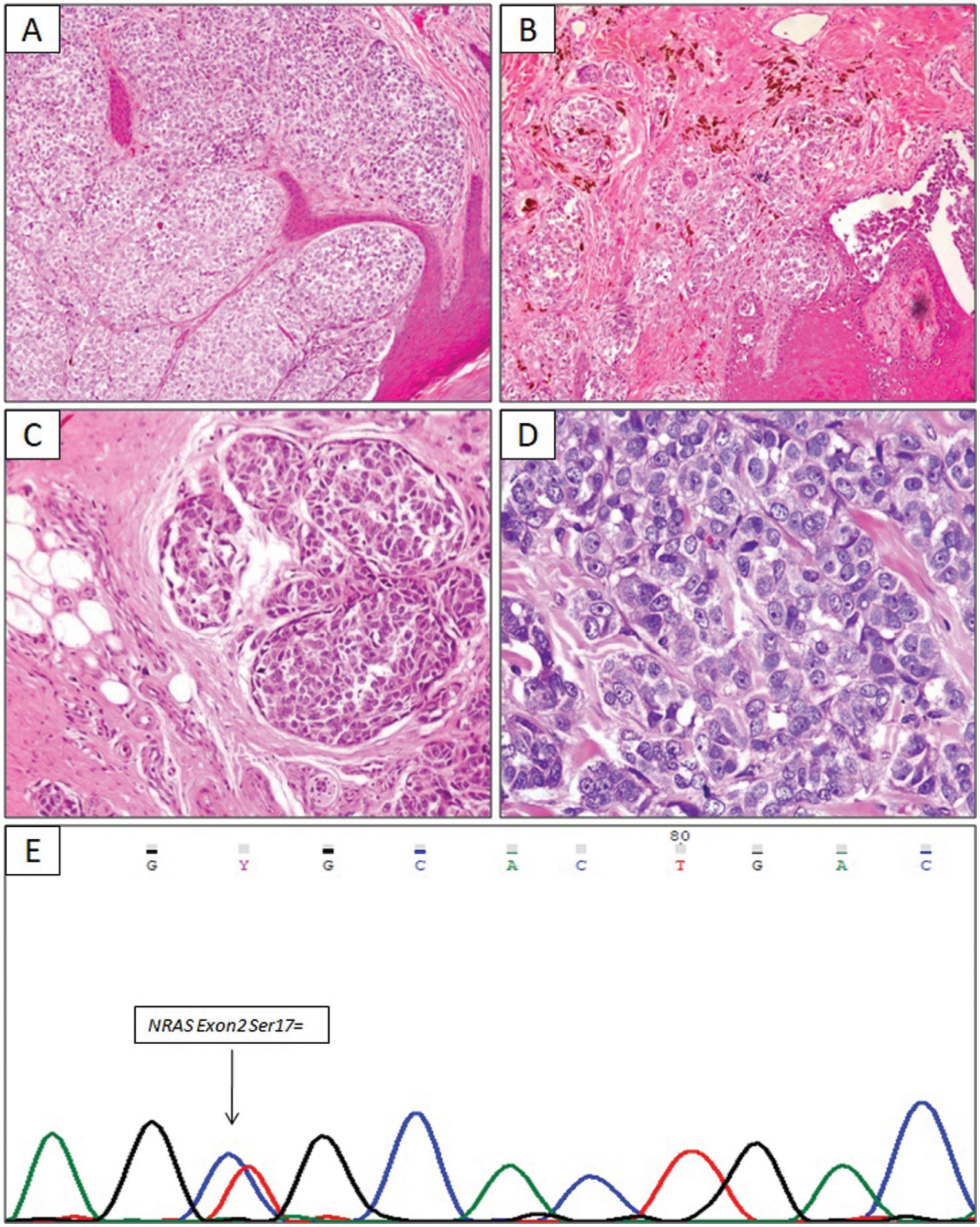

Fig. 5 Acral lentiginous melanoma, *NRAS* mutant (A–E): (**A**) Tumor in the form of nodules in the epidermis, papillary dermis. Hematoxylin and eosin (H&E) x100. (**B**) Tumor shows distinct melanin pigment. H&E x100. (**C**) Tumor composed of polygonal cells infiltrating subcutaneous tissue. H&E x200. (**D**) Tumor showing prominent nucleoli. H&E x400. (**E**) Electropherogram showing *NRAS* Exon 2Ser17= mutation.

Among 8 studies reported from Asia over the last 5 years, only two studies included evaluation of *BRAF* and *NRAS* alterations in MM.[28,29] This study is the first of its kind from India exploring the role of both *BRAF* and *NRAS* mutations in MM.

It is noteworthy that the frequencies of mutations underlying melanomas in this study were similar to those from Taiwan and Indonesia. Likewise, the frequency of *NRAS* mutation was similar to that observed in studies from other Asian countries.[28,29]

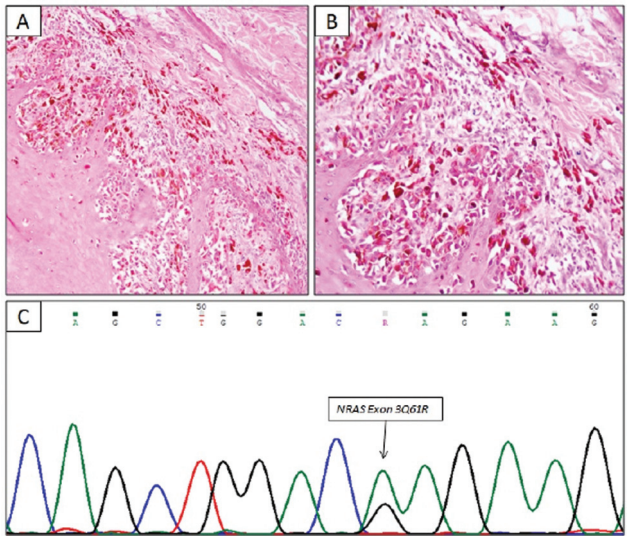

Fig. 6 Acral lentiginous melanoma, *NRAS* mutant (A–C): (**A**) Tumor involving epidermis and papillary dermis with prominent junctional activity, pagetoid spread and melanin pigment. Hematoxylin and eosin (H&E) x100. (**B**) Tumor cells with intracellular melanin at dermo epidermal junction and papillary dermis. H&E x400. (**C**) Electropherogram showing *NRAS* Exon 3 p.Gln61Arg mutation.

Table 4 Geographical distribution of melanoma subtype and molecular alterations observed in the cohort

Classification		Region			
	Type	North	South	East	West
	SSM	0	0	1	1
	ALM	2	1	5	6
	Nodular M	7	2	5	15
	Conjunctival M	1	0	0	3
	Mucosal M	5	2	17	9
	Desmoplastic M	0	0	0	2
	MM with unknown primary	0	0	1	3
BRAF	Mutant	1	0	7	4
	Wildtype	12	5	18	27
NRAS	Mutant	0	1	2	3
	Wildtype	8	1	14	9

Abbreviations: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma.

Few studies have identified *BRAF*-Fusions in about 4 to 6% of cases in "pan-negative" (negative for common mutations) melanomas.[42] These fusions present an alternate mechanism of constitutive activation of the MAPK pathway due to the lack of the 5′ auto-inhibitory domain of *BRAF* gene. There have been reports describing the presence of *BRAF* Fusion viz; AGAP3-*BRAF* in MM patients being treated using *BRAF* inhibitors. The *BRAF* fusions have been reported in both *BRAF* V600E mutant and wildtype cases. *BRAF* Fusion, irrespective of the use of *BRAF* inhibitors, plays an important role in the clonal selection of fusion- positive melanoma tumor cells.[42,43] Understanding the role of these *BRAF* fusions

Table 3 Clinicopathological characteristics of cases harboring *BRAF and NRAS* mutation

Mutation type		Tumor type	Gender	Age	Site
BRAF	V600E	ALM ($n=1$)	Female ($n=2$) Male ($n=5$)	~57.1	Stomach
		NMM ($n=1$)			Foot
		MMM ($n=3$)			Upper back
		SSM ($n=1$)			Rectum
		MUP ($n=1$)			Inguinal lymph node
BRAF	A594T	MMM ($n=1$)	Male ($n=2$)	~63	Rectum
		ALM ($n=1$)			Thumbnail bed
	T599=	NMM ($n=1$)	Male	58	Foot
		CMM ($n=1$)	Female	57	Left eye
	V600K	NMM ($n=1$)	Male	47	Neck
	Q612P	ALM ($n=1$)	Male	73	Thumbnail bed
NRAS	A59V	MUP ($n=1$)	Male	57	Inguinal lymph node
	G13D	SSM ($n=1$)	Female	52	Inguinal lymph node
		MMM ($n=1$)	Female	41	Sinonasal
	S17=	ALM ($n=1$)	Male	68	Left heel
	Q61R	ALM ($n=1$)	Female	43	Left heel
		MMM ($n=1$)	Male	39	Stomach

Abbreviations: ALM, acral lentiginous melanoma; CMM, conjunctival mucosal melanoma; IHC, immunohistochemistry; MMM, malignant mucosal melanoma; MUP, MM of unknown primary; NMM, nodular mucosal melanoma; SSM, superficial spreading melanoma.

Table 5 Comparison of incidences of molecular alterations melanoma reported across Asia in the last 5 years

Country	Sample size	Melanoma	*BRAF*	*NRAS*	Method of detection	Reference
Japan	80	Cutaneous malignant melanoma	41.80%	–	Real-time PCR	Yamazaki et al[31] 2015
Russia	90	Primary melanoma	43.3%	–	Real-time PCR	Aksenenko et al[32] 2015
Taiwan	119	Malignant melanoma	14.30%	10.10%	Sanger sequencing	Sheen YS et al[28] 2016
Korea	52	Acral	6.40%	4.30%	NGS	Shim et al[29] 2017
Uyghur, China	60	Malignant melanoma	23%	–	Real-time PCR, sequencing	Kang et al[1] 2018
Java, Indonesia	40	Cutaneous malignant melanoma	10%	–	Real-time PCR	Rinonce et al[30] 2019
India	70	Malignant melanoma	30%	–	Pyrosequencing	Ahmad et al[19] 2019
India	88	Malignant melanoma	16.21%	15.71%	Real-time PCR, sequencing	Present study

Abbreviations: NGS, next-generation sequencing; PCR, polymerase chain reaction.

and mutations is very important to plan the treatment strategies for the patients, that is, whether to use *BRAF* inhibitors alone or in combination with mitogen-activated protein kinase kinase (MEK) inhibitors.[43]

While the strength of this study is that it is the first comprehensive study from the Indian subcontinent exploring the role of *BRAF* and *NRAS* mutation spectrum in MM, there were certain limitations such as the type of mutation testing platforms used and the sensitivity of the assay used to detect these alterations. Another limitation was testing our cases with the BRAF antibody (monoclonal, VE1), which seems to be a promising surrogate for *BRAF* mutation, especially in view of its high sensitivity and specificity.[44,45] We intend to further test our cases with BRAF VE1 antibody in our subsequent studies.

Conclusion

In conclusion, this study is the first and the largest study to include *BRAF* and *NRAS* alterations in melanoma subtypes observed among the Indian population. Nodular MM was the commonly observed subtype of MM, associated with *BRAF* alterations. *NRAS* alterations were more frequent in cases of ALM. Mucosal melanoma was the most common noncutaneous melanoma in this study cohort. A larger sample size, with more extensive molecular markers, such as *NF-1, KIT*, and *BRAF* fusions would yield additional information on the disease manifestation.

Authors' Contributions

O.S. contributed to conceptualization, methodology, administration. V.V. helped in data curation, writing-original draft preparation. M.G. contributed to methodology and investigation. P.B. helped in investigation and supervision. N.K. contributed to analysis and investigation. G.W. and M.R. were involved in investigation. B.R. and E.S. reviewed and edited the manuscript. S.D. helped in administration and visualization.

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Conflict of Interest

None declared.

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