

BRAF mutation status in primary and metastatic melanomas in two regions with differing potential ultraviolet radiation exposure

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Summary

Background. Melanoma is seen as a heterogeneous molecular entity, with solar ultraviolet radiation (UVR) and *BRAF* mutation status being important determinants.

Aim. To study primary and metastatic melanomas from two UVR-distinct regions to elucidate correlations between prognostic predictors, UVR and *BRAF* mutation status.

Methods. Extended *BRAF* testing for 9 mutations was obtained for 95 primary melanomas [Lebanon (LB) $n = 55$, Pakistan (PK) $n = 40$] and 65 metastatic melanomas (LB $n = 36$, PK $n = 29$). Collected data included patient age and sex, melanoma size and anatomical location, prognostic parameters and solar elastosis grade for primary melanomas. For metastatic melanomas, site of metastasis, magnitude of necrosis and degree of pigmentation were assessed. Cumulative 21-year averages of potential UVR exposure for Lebanon (110 kJ/m²/year) and Pakistan (128 kJ/m²/year) were derived from the National Center for Atmospheric Research databases.

Results. *BRAF* mutation status was obtained for 146/160 cases (91.3%). Overall mutation rate was 24/88 (27.3%) in primary and 25/58 (43.1%) in metastatic melanoma. V600E was the predominant mutation in 21/24 (87.5%) of primary and 23/25 (92%) of metastatic melanomas. A 60% discordant mutation rate was identified; of three patients, two lost the mutation in the metastasis and one gained it. The relative incidence of *BRAF* mutation with potential UVR exposure showed a similar trend in primary (low vs. high UVR: 32.1% vs. 20.0%) and metastatic (57.1% vs. 21.7%) melanomas ($P < 0.05$). Predictors of *BRAF* mutations were trunk location and epithelioid and mixed cytology for primary and subcutaneous metastasis, low UVR exposure and absence of pigmentation for metastatic melanomas ($P < 0.05$). *BRAF*-positive status in primary melanomas was predicted by multivariate binary logistic regression with reasonable accuracy (C-statistic = 0.67, 95% CI 0.530–0.81 with one independent predictor, namely, epithelioid cytology (OR = 5.11, 95% CI 1.38–8.88, $P = 0.01$). In metastatic melanomas, high UVR (OR = 0.21, 95% CI 0.06–0.07; $P < 0.01$) was an independent negative predictor of *BRAF* mutation.

Conclusions. We have documented the rate of different *BRAF* mutation types in a Lebanese and Pakistani cohort, and assessed correlations with prognostic markers and potential UVR exposure.

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Introduction

Cutaneous melanoma is an aggressive tumour, whose incidence is rising in western countries. Patients with metastatic disease at presentation are resistant to current systemic treatments, and the 5-year survival rate is < 10%.¹ Consequently, there is great interest in the development of novel therapies targeting specific molecular events in melanoma progression.

BRAF is a proto-oncogene, specifically a serine/threonine kinase, and is a mediator in the RAS/RAF/MEK/ERK pathway. This pathway normally affects cellular proliferation and differentiation, and triggers apoptosis. *BRAF* mutation is present in about 66% of malignant melanomas, and has received special attention. In fact, selective *BRAF* inhibitors are showing promising results in current clinical trials.² Most *BRAF* mutations occur by replacement of thymine by adenine at codon V600E of exon 15. This leads to the change of valine 600 to glutamic acid. In consequence, *BRAF* constitutively activates MEK/ERK, possibly contributing to tumourigenesis.^{3,4}

BRAF mutation is present in up to 80% of melanocytic naevi, most of which never progress to melanoma.⁵ Studies have suggested inhibition of this pathway in naevi by *BRAF* V600E-induced synthesis and secretion of IGFBP7, a tumour suppressor protein, leading to cellular senescence and apoptosis.⁶ However, this tumour suppression mechanism is absent in *BRAF* V600E-positive melanomas, where IGFBP7 expression is lost.⁷

Knowing the triggers of *BRAF* mutation, which are as yet ill-defined, may aid in preventing the disease. Ultraviolet radiation (UVR), a major factor in melanoma pathogenesis, has been proposed to have an indirect effect on *BRAF* mutational status.⁸ This mutation is most commonly present in those melanoma subtypes that are associated with sun exposure, namely superficial spreading and nodular melanomas.^{9–11} In addition, the mutation is most prevalent on intermittently sun-exposed skin of young people, and occurs at much lower frequencies in mucosal melanomas and in cancers of sun-protected organs.¹¹ Therefore, an interaction between UVR and *BRAF* mutation seems to exist, yet the direct causal relationship has been questioned because these mutations are not typical UVR signature mutations.

The primary aims of our study were: to highlight the epidemiology of *BRAF* mutation in primary and metastatic melanoma in the Near East region, to shed light on different *BRAF* mutation types by performing extended *BRAF* testing, to correlate clinical and pathological prognostic factors with *BRAF* mutation status,

and to investigate the association between UVR and *BRAF* mutation.

Methods

Sample selection and clinical data

This study was approved by the institutional review board of the American University of Beirut Medical Center (PALM.IK.02). We collected 160 formalin-fixed, paraffin wax-embedded tissue blocks from tumours that had been histologically diagnosed as cutaneous melanomas between 1996 and 2011. These were collected from the Pathology and Dermatology archives at the American University of Beirut, Lebanon (55 primary melanomas from 54 patients and 36 metastatic melanomas from 32 patients) and Shaukat Khanum Memorial Cancer Hospital and Research Centre, Lahore, Pakistan (40 primary melanomas from 40 patients and 29 metastatic melanomas from 29 patients). For primary melanomas, clinical parameters including patient age and sex, and melanoma size and anatomical location were recorded. For metastatic melanomas, the size and site of distant metastasis (lymph node, subcutaneous, lung, liver, other) were documented. Cases with incomplete clinical data or insufficient material for PCR were excluded from the study.

Potential solar ultraviolet exposure rates for each geographical location

To assess the effect of long-term potential environmental exposure to UVR and its possible role in the induction of various biological chemical processes including *BRAF* mutation, the distributions of monthly mean surface level of erythemally effective UVR were calculated for the period 1979–2000, using the Tropospheric Ultraviolet Visible radiative transfer model (Denver, CO, USA)¹² with inputs from ozone instruments [Total Ozone Mapping Spectrometers (TOMS), aboard the Nimbus-7, Meteor-3, and Earth probes]. These were averaged, and were found to be 110 kJ/m²/year for Lebanon and 128 kJ/m²/year for Pakistan. The time frame (1979–2000) selected to average the UVR allows enough sunlight accumulation to elucidate the UV–*BRAF* interaction.

Histological evaluation

Each haematoxylin and eosin-stained slide was examined by two pathologists (IK and CYM), and the histological type of melanoma was determined (superficial

spreading melanoma, nodular melanoma, acral lentiginous melanoma, lentigo maligna melanoma). The following histological and prognostic parameters of primary melanoma were recorded: Breslow thickness, Clark level, mitotic rate/mm², predominant cytology (epithelioid, spindle or mixed population), tumour-infiltrating lymphocytes (absent, 'nonbrisk' and 'brisk') and the margin status. The following features were categorized as 'present' or 'absent': radial growth phase, vertical growth phase, regression, ulceration, lymphovascular and perineural invasion, histological satellites and associated naevi.

In order to find potential morphological predictors of *BRAF* mutation, the following parameters were recorded in primary melanomas.¹³

- *Upward scatter of intraepidermal melanocytes.* The upward scatter of intraepidermal melanocytes was recorded as absent (all the melanocytes are present at the dermoepidermal junction, with only rare ones in higher epidermal layers), mild (75–100% of melanocytes are present at the dermal-epidermal junction, and some are present in higher epidermal layers), moderate (intraepidermal melanocytes are equally present at the dermoepidermal junction and in higher layers of the epidermis) and prominent [most (> 50%) of the intraepidermal melanocytes are situated in upper layers of the epidermis].
- *Nesting of intraepidermal melanocytes.* Nesting was reported as absent (almost all intraepidermal melanocytes are present as single cells, nests are rare), mild (intraepidermal melanocytes are predominantly present as single cells, < 25% as nests), moderate (25–50% of intraepidermal melanocytes are arranged in nests) and prominent (more than 50% of intraepidermal melanocytes are arranged in nests).
- *Circumscription.* Circumscription was recorded as abrupt or gradual, according to the transition of the intraepidermal part of the tumour to normal skin at the tumour periphery.
- *Epidermal contour.* The epidermal contour involved by the radial growth phase compared with the adjacent normal epidermis was classified as atrophic, normal or hyperplastic.
- *Nuclear size of melanocytes.* The nuclear size was compared with that of basal cells and recorded as: small (smaller than a basal cell nucleus), medium (1–2 times the size of a basal cell nucleus) or large (more than twice the size of a basal cell nucleus).
- *Pigmentation.* Pigmentation was reported as absent (no pigment), faint (pigmentation is visible at high power), moderate (pigmentation is visible at low

power) or severe (pigmentation hiding melanocytic nuclei).

To assess the effect of cumulative UVR exposure on *BRAF* mutation more thoroughly, the degree of sun damage reflected by solar elastosis was graded as absent, intermittent (patchy solar elastosis) or severe (coalescent solar elastosis).

The histological parameters recorded for metastatic melanomas were the following: predominant cytology, location of the metastasis within the lymph node (absent, cortical, medullary or combined), necrosis (absent, focal or extensive) and the mitotic rate/mm². Tumour-infiltrating lymphocytes were reported for all metastatic sites excluding lymph nodes. Pigmentation was recorded as absent, focal (involving ≤ 10% of the lesion) and heavy (involving > 10% of the lesion). Anaplasia, in-transit metastasis and extranodal extension were reported as 'absent' or 'present'.

DNA extraction and extended *BRAF* mutational testing

All the included cases had one distinct morphological clone and the slides of every case were reviewed and marked for microdissection. DNA was extracted from formalin-fixed paraffin wax-embedded (FFPE) tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). *BRAF* genotyping was performed by PCR followed by reverse hybridization. Briefly, a DNA fragment spanning *BRAF* codons 600–601 was amplified and biotinylated by PCR using primers described in previous publication.¹⁴ Amplicons were hybridized for 30 min at 45 ± 0.5 °C to a membrane teststrip presenting a parallel array of allele-specific oligonucleotides for each of the nine *BRAF* mutations assessed: V600A (c.1799T>C), V600D (c.1799_1800TG>AT), V600E (c.1799T>A), V600E (c.1799_1800TG>AA), V600G (c.1799T>G), V600K (c.1798_1799GT>AA), V600M (c.1798G>A), V600R (c.1798_1799GT>AG) and K601E (c.1801A>G). After a series of stringent washes, specifically bound PCR fragments were detected using a streptavidin–alkaline phosphatase conjugate and colour substrates (BCIP/NBT). The entire hybridization and detection procedure was fully automated and carried out using a temperature-controlled teststrip processor (profiBlot II T 48; Tecan, Groedig, Austria). Reference DNA samples previously typed by direct DNA sequencing were available for all SNPs, and were used for performance control of the assay using serial dilutions of *BRAF*-mutated cell lines. The assay was shown to detect 1% mutated DNA in a background of wild-type DNA.

Statistical analysis

Continuous variables were analyzed by *t*-test or Mann–Whitney rank sum test as appropriate. Categorical variables were analyzed using χ^2 test. A two-tailed *P* value of < 0.05 was required for statistical significance. Independent predictors of *BRAF*-positive status were derived by multivariate binary logistic regression (backward selection). A univariate significance level of *P* < 0.2 was used for inclusion, and factors were retained if they were significant at *P* < 0.05. Analyses were performed using SPSS (v19; IBM Inc., Somers, NY, USA).

Results

Population demographics and clinical parameters in relation to *BRAF* somatic mutation status

BRAF mutation status was obtained by reverse hybridization for 146/160 cases (91.3%). The overall rate of *BRAF* mutation was 49/146 (33.6%). The mutation was present in 24/88 (27.3%) of primary melanomas and 25/58 (43.1%) of metastatic melanomas.

V600E was the predominant type of *BRAF* mutation, present in 44/49 (89.8%) of cases. It was found in 21/24 (87.5%) of primary melanomas and 23/25 (92%) of metastatic melanomas, and 1 primary melanoma case had the V600E c.1799–1800 TG>AA *BRAF* mutation. The second most common mutation in our population was V600K, with an overall frequency of 5/49 (10.2%). It was present in 3/24 (12.5%) of primary melanomas and 2/25 (8%) of metastatic melanomas.

The ages of the patients with primary melanoma ranged from 0.33 to 103 years (mean 55.2 years). Patients with metastatic melanoma were aged 25–87 years (mean 53.9 years). The mutation rate was not affected by age or size in either of the geographic locations, as noted in Tables 1 and 2.

For metastatic melanoma, the lesions were predominantly located in lymph nodes (34/58, 58.6%). The rest of the lesions were distributed as follows: subcutaneous 22.4% (13/58), lung 6.9% (4/58), liver 3.4% (2/58), brain 5.2% (3/58) and breast 3.4% (2/58). Subcutaneous metastasis was the only significant positive predictor of *BRAF* mutation in metastatic melanoma (*P* < 0.05).

Histological findings and *BRAF* somatic mutation status

The mutation status was correlated with the histopathological parameters of the corresponding primary and metastatic tumours. Significant positive predictors

of *BRAF* mutation in primary melanoma were epithelioid and mixed cytologies (*P* < 0.05; Tables 1 and 2). Marginally significant predictors of the mutation were Clark level 4, perineural invasion and the absence of tumour-infiltrating lymphocytes (*P* < 0.10). For metastatic melanoma, the absence of pigmentation was a significant positive predictor of *BRAF* mutation (*P* < 0.05). None of the morphological parameters studied in primary melanoma (upward scatter of cells, nesting, circumscription, epidermal contour, nuclear size and pigmentation) were able to discriminate significantly between *BRAF*-positive and *BRAF*-negative tumours (Fig. 1).

Effects of potential solar exposure on *BRAF* somatic mutation in primary and metastatic melanomas

We tested the potential UVR exposure by three indirect and direct methods to validate the UV–*BRAF* interaction (Tables 1–4): (i) calculation of the erythemally effective UVR by latitude and longitude for each city studied for the 21 years prior to collecting the cases studied; (ii) histological quantification of cumulative sun damage through solar elastosis; and (iii) correlation of the anatomical location of the naevi with the expected sun exposure trend for that particular anatomical location (i.e. chronic, intermittent or unexposed).

For both primary and metastatic melanomas, patients living in regions with lower UVR exposure had significantly higher *BRAF* mutation frequencies than those living in more exposed regions (primary melanomas: Lebanon 32.1% vs. Pakistan 20%; metastatic melanomas: Lebanon 57.1% vs. Pakistan 21.7%). However, UVR was a significant negative predictor of *BRAF* mutation only for metastatic melanoma (*P* = 0.008).

Solar elastosis, a histological marker of cumulative UVR exposure, was detected in 17/64 (26.6%) of *BRAF*-negative primary melanomas (7 intermittent solar elastoses and 10 coalescent solar elastoses) and in 4/24 (16.7%) of *BRAF*-positive primary melanomas (1 intermittent solar elastosis and 3 coalescent solar elastoses). However, the difference in *BRAF* mutation rates between lesions with or without solar elastosis was not statistically significant (*P* = 0.5).

Anatomical location has been traditionally divided into three categories: sun-protected, intermittently sun-exposed (e.g. trunk) or constantly sun-exposed locations.¹³ In our cohort, the leg (38/88, 43.2%) was the predominant location of primary melanoma. The remaining lesions were distributed as follows: trunk

Table 1 Overall *BRAF* somatic mutation status: clinical and microscopic findings in 88 cases of primary melanoma.

Variable	BRAF-negative		BRAF-positive		P
	n	%	n	%	
Samples	64	72.7	24	27.3	–
Clinical parameters					
Age, years	55.9	–	49.1	–	0.30
Lesion size, mm	21.7	–	17.8	–	0.42
Sex					0.32
Female	26	40.6	7	29.2	
Male	38	59.4	17	70.8	
Anatomical location					
Head and neck	13	20.3	2	8.3	0.18
Arm	11	17.2	4	16.7	0.95
Leg	29	45.3	9	37.5	0.51
Trunk	11	17.2	9	37.5	0.04
Histological parameters					
Breslow thickness (mm)	5.6	–	4.0	–	0.13
Multiple primary lesions					0.30
Absent	62	96.9	22	91.7	
Present	2	3.1	2	8.3	
Mitotic rate/mm ²	7.4	–	7.1	–	0.74
Histological type					0.30
SSM	31	48.4	16	66.7	
Lentigo maligna melanoma	0	0	0	0	
Nodular melanoma	17	26.6	6	25	
Acral lentiginous melanoma	14	21.9	2	8.3	
Clark level					
1	2	3.1	0	0	0.38
2	9	14.1	2	8.3	0.47
3	3	4.7	2	8.3	0.51
4	30	46.9	16	66.7	0.10
5	20	31.2	4	16.7	0.17
Predominant cytology					
Epithelioid	37	57.8	21	87.5	0.01*
Spindle	4	6.3	0	0	0.21
Mixed	23	35.9	3	12.5	0.03*
Solar elastosis					0.54
Absent	47	73.4	20	83.3	
Intermittent	7	10.9	1	4.2	
Severe	10	15.6	3	12.5	
Necrosis					0.47
Absent	57	89.1	20	83.3	
Present	7	10.9	4	16.7	
Ulceration					0.46
Absent	29	45.3	13	54.2	
Present	35	54.7	11	45.8	
Regression					0.16
Absent	59	92.2	24	100	
Present	5	7.8	0	0.0	
Perineural invasion					0.09*
Absent	57	89.1	24	100	
Present	7	10.9	0	0.0	
Vertical growth phase					0.82
Absent	12	18.8	4	16.7	
Present	52	81.3	20	83.3	

Table 1. continued

Variable	BRAF-negative		BRAF-positive		P
	n	%	n	%	
Radial growth phase					0.27
Absent	18	28.1	4	16.7	
Present	46	71.9	20	83.3	
Microscopic satellitosis					0.57
Absent	64	100	24	100	
Present	0	0	0	0.0	
Tumour-infiltrating lymphocytes					0.07*
Absent	8	12.5	0	0	
Nonbrisk	50	78.1	20	83.3	0.59
Brisk	6	9.4	4	16.7	0.34
Vascular invasion					0.58
Absent	56	87.5	22	91.7	
Intermittent	8	12.5	2	8.3	
Surgical margins					0.53
Clear	42	65.6	14	58.3	
Involved	22	34.4	10	41.7	
UV region					0.21
Low	36	56.3	17	70.8	
High	28	43.8	7	29.2	

*Variables presented as average. Significant at $P < 0.05$. SSM, superficial spreading melanoma; UV, ultraviolet.

22.7% (20/88), head and neck 17% (15/88) and arm 17% (15/88). *BRAF* mutational rate in primary melanomas varied with anatomical location: primary melanomas located on the trunk and leg harboured the highest rate of *BRAF* mutation (trunk 37.5%, leg 37.5%). Trunk location was the only anatomical site significantly predictive of positive *BRAF* mutational status ($P < 0.05$).

Status of *BRAF* mutations in patients with multiple specimens

Eight patients had multiple specimens, five of whom had primary and corresponding metastatic melanomas (Table 5). Patients 1, 2 and 3 had one primary lesion and its corresponding metastasis; patient 4 had two primary lesions and one corresponding metastasis; patient 5 had one primary lesion and two corresponding metastases; and patient 6 had two primary melanomas. Multiple metastatic lesions only were tested in patients 7 ($n = 2$) and 8 ($n = 3$).

We investigated all five cases of primary and their corresponding metastatic melanomas. Three of the five (60%) had discordant mutation status: two patients had lost the mutation in the metastasis and one gained it. The remaining two patients had the wild-type

Table 2 Overall *BRAF* somatic mutation status: clinical and microscopic findings in 58 cases of metastatic melanoma.

Variable	BRAF-negative		BRAF-positive		P
	n	%	n	%	
Samples	33	56.9	25	43.1	
Clinical parameters					
Age, years	52.9	–	56.4	–	0.35
Lesion size, mm	26.8	–	25.1	–	0.54
Sex					
Female	15	45.5	10	40	0.68
Male	18	54.5	15	60	
Metastatic site					
Lymph node	21	63.6	13	52	0.37
Subcutaneous	4	12.1	9	36	0.03
Lung	2	6.1	2	8	0.77
Liver	2	6.1	0	0	0.21
Other	4	12.1	1	4	0.28
Histological parameters					
Mitotic rate/mm ²	14.8	–	11	–	0.57
Lymph-node metastasis					
Absent	11	33.3	12	48	0.52
Cortical	3	9.1	2	8	
Medullary	0	0	0	0	
Combined	19	57.6	11	44	
Extranodal extension					
Absent	17	51.5	17	68	0.21
Present	16	48.5	8	32	
Pigmentation					
Absent	14	42.4	18	72	0.03
Focal	10	30.3	4	16	
Heavy	9	27.3	23	92	
Tumour-infiltrating lymphocytes					
Absent	21	63.6	14	56	0.56
Nonbrisk	3	9.1	0	0	
Brisk	9	27.3	11	44	
Predominant cytology					
Epithelioid	23	69.7	19	76	0.45
Spindle	2	6.1	0	0	
Mixed	8	24.2	6	24	
Anaplasia					
Absent	12	36.4	3	12.0	0.10
Present	21	63.6	22	88.0	
Necrosis					
Absent	8	24.2	9	36	0.62
Focal	16	48.5	10	40	
Extensive	9	27.3	6	24	

Variables presented as average. *Significant at $P < 0.05$.

genotype in both the primary tumour and the corresponding metastasis sites.

Independent predictors of *BRAF* mutation

A *BRAF*-positive mutation status in primary melanomas was reasonably well predicted by multivariate binary logistic regression (C-statistic = 0.67, 95% CI 0.53–0.81) by one independent predictor, namely,

epithelioid cytology (OR = 5.13, 95% CI 1.38–8.88, $P = 0.01$). UVR was not an independent predictor of *BRAF* mutation. In metastatic melanomas, the only independent predictor was high UVR (OR = 0.21, 95% CI 0.06–0.07; $P < 0.01$).

Discussion

Molecularly targeted therapy has shown promising results in early trials for disseminated melanoma. In August 2011, a specific *BRAF* inhibitor (vemurafenib) was approved by the US Food and Drugs Administration for the treatment of V600E mutant melanomas.^{15,16} Since then, *BRAF* mutational rate has been studied extensively in primary lesions. However, reports of its frequency in metastatic tumours are limited, and available data are based on homogeneous white and East Asian populations. Our study is the first of its kind performed on populations from regions in the Near East with different potential annual erythemal UVR exposure. It is also unique in performing extended *BRAF* testing for 9 different mutations on a large series of both primary (95 cases) and metastatic (65 cases) melanomas. This allowed us to detect the types of *BRAF* mutation peculiar to our populations and to correlate this mutation with the usual prognostic clinicopathological parameters. The correlation of *BRAF* mutation with solar UVR exposure was also analyzed, based on the degree of solar elastosis, the anatomical location of the tumour, and erythemal UVR measurements derived from the databases of the National Center for Atmospheric Research.¹²

***BRAF* somatic mutation rate in primary and metastatic melanomas**

Previous reports on the rate of *BRAF* mutation in primary melanoma have ranged from 15% to 80%.^{5,16} This variation may be due to different proportions of melanoma types, disease stages, detection methods and possibly, the regions of the studied cohorts. In our population, the *BRAF* mutational rate (24/88, 27.3%) was intermediate between rates of cohorts from East Asia [16/109 (15%) from China]¹⁷ and western populations,⁹ [28/97 (29%) from Germany,¹⁸ 112/251 (45%) from Australia,¹⁹ 142/302 (47%) from California, USA,¹³ 116/213 (54%) from Sweden²⁰ and 29/44 (65.9%) from Texas, USA].²¹

Very few reports on the mutational rate in metastatic melanoma are available in the literature. A notably higher figure in metastasis (39/68, 57%)

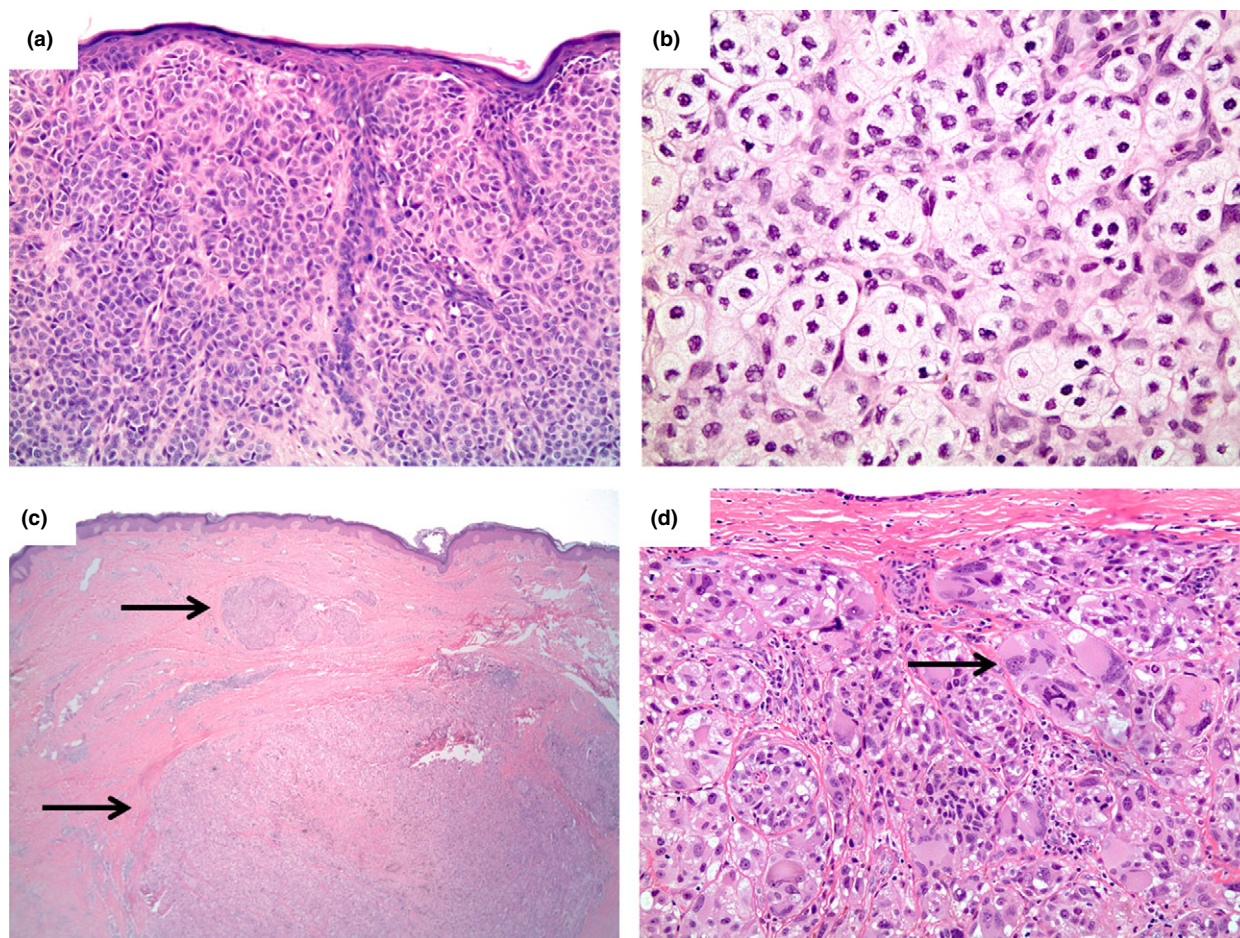


Figure 1 *BRAF*-positive primary amelanotic melanomas with (a) nested pattern, epithelioid cytology and (b) balloon cell changes. Haematoxylin and eosin, original magnification (a) $\times 100$; (b) $\times 400$. (c) *BRAF*-positive metastatic melanomas situated in the subcutaneous tissue (arrow), which (d) lack melanin pigment and exhibit epithelioid cytology and anaplastic changes (arrow) original magnification (c) $\times 20$; (d) $\times 200$.

compared with primary melanoma (18/59, 31%) was given by Shinozaki *et al.*²² Pollock *et al.*⁵ found mutations in 68% (41/60), and Gorden *et al.*²³ found mutation in 40% (31/77) of metastatic melanoma cases. Our results were comparable with those of Shinozaki *et al.*, with a 43.1% mutational rate in metastatic vs. 27.3% in primary melanomas. The higher rates of *BRAF* mutations in metastases compared with primary melanomas are a rationale for anti-RAS/RAF treatments in metastatic disease.

We also compared the mutational status in primary and matching metastatic melanomas. Previous studies have yielded conflicting results. In a study of 51 matched pairs of primary and metastatic melanomas, Omholt *et al.* demonstrated that the mutation present in a primary tumour is always preserved in the corresponding metastasis. Furthermore, all the metastases

corresponding to primary lesions with the wild-type gene did not have the mutation, with the exception of two patients who acquired the mutation in their metastases.²⁴ Shinozaki *et al.* examined 13 pairs of primary and their associated metastases. In 5 out of the 13 pairs (38.4%), the mutation was present only in the metastases.²² Our findings were even more variable. Two patients had the *BRAF* mutation in the primary lesion and lost it in the metastasis (2/5, 40%). One patient had a wild-type primary tumour with a mutated metastasis (1/5, 20%). Two *BRAF*-negative primary and concomitant metastases were also present. In view of the discordant *BRAF* mutation status between primary and corresponding metastases and the limited sample size, we support further experimental evidence for testing *BRAF* in the metastasis prior to treatment.

Table 3 Correlation between BRAF status, clinical and microscopic parameters in regions with distinct potential annual erythemal UV exposure for primary melanoma cases.

Primary melanoma variables	Lebanon (n = 53)					Pakistan (n = 35)				
	BRAF-negative		BRAF-positive		P	BRAF-negative		BRAF-positive		P
	n	%	n	%		n	%	n	%	
Samples	36	67.9	17	32.1		28	80	7	20	0.02
Clinical parameters										
Sex	0.39									
Female	21	58.3	12	70.6		17	60.7	5	71.4	0.60
Male	15	41.7	5	29.4		11	39.3	2	28.6	
Anatomical location										
Head and neck	11	30.6	1	5.9	< 0.05	2	7.1	1	14.3	0.55
Arm		22.2	4	23.5	0.92	3	10.7	0	0	0.37
Leg	9	25.0	6	35.3	0.44	20	71.4	3	42.9	0.15
Trunk	8	22.2	6	35.3	0.31	3	10.7	3	42.9	0.04
Histological parameters										
Histological type	0.436									
Superficial spreading melanoma	22	61.1	12	70.6		11	39.3	4	57.1	0.68
Lentigo maligna melanoma	0	0	0	0		0	0	0	0	
Nodular melanoma	7	19.4	4	23.5		10	35.7	2	28.6	
Acral lentiginous melanoma	7	19.4	1	5.9		7	25.0	1	14.3	
Clark level										
1	1	2.8	0	0.0	0.49	1	3.6	0	0	0.61
2	8	22.2	2	11.8	0.36	1	3.6	0	0	0.61
3	2	5.6	0	0.0	0.32	1	3.6	2	28.6	0.04
4	16	44.4	12	70.6	0.08	14	50.0	4	57.1	0.74
5	9	25.0	3	17.6	0.55	11	39.3	1	14.3	0.21
Predominant cytology										
Epithelioid	21	58.3	15	88.2	0.03	16	57.1	6	85.7	0.16
Spindle	1	2.8	0	0.0	0.488	3	10.7	0	0	0.37
Mixed	14	38.9	2	11.8	< 0.05	9	32.1	1	14.3	0.35
Solar elastosis	0.66									
Absent	23	63.9	14	82.4	0.39	24	85.7	6	85.7	
Intermittent	5	13.9	1	5.9		2	7.1	0	0	
Severe	8	22.2	2	11.8		2	7.1	1	14.3	
Ulceration	0.87									
Absent	22	61.1	10	58.8		7	25.0	3	42.9	0.35
Present	14	38.9	7	41.2		21	75.0	4	57.1	
Regression	0.32									
Absent	34	94.4	17	100.0		25	89.3	7	100	0.37
Present	2	5.6	0	0.0		3	10.7	0	0	
Perineural invasion	0.07									
Absent	30	83.3	17	100.0		27	96.4	7	100	0.61
Present	6	16.7	0	0.0		1	3.6	0	0	
Vertical growth phase	0.14									
Absent	11	30.6	2	11.8		1	3.6	2	28.6	0.04
Present	25	69.4	15	88.2		27	96.4	5	71.4	
Radial growth phase	0.70									
Absent	8	22.2	3	17.6		10	35.7	1	14.3	0.26
Present	28	77.8	14	82.4		18	64.3	6	85.7	
Microscopic satellitosis										
Absent	36	100.0	17	100.0	–	28	100.0	7	100	–
Present	0	0.0	0	0.0		0	0.0	0	0	
Tumour-infiltrating lymphocytes										
Absent	6	16.7	0	0.0	0.15	2	7.1	0	0	0.47
Nonbrisk	24	66.7	15	88.2	0.01	26	92.9	5	71.4	0.11
Brisk	6	16.7	2	11.8	0.64	0	0.0	2	28.6	0.01

Significant at $P < 0.05$. UV, ultraviolet.

Table 4 Correlation between BRAF status, clinical and microscopic parameters in regions with distinct potential annual erythemal ultraviolet exposure for metastatic melanoma cases.

Metastatic melanoma variables	Lebanon (n = 53)				P	Pakistan (n = 35)				P			
	BRAF-negative		BRAF-positive			BRAF-negative		BRAF-positive					
	n	%	n	%		n	%	n	%				
Samples	15	42.9	20	57.1	–	18	78.3	5	21.7	0.01			
Clinical parameters													
Sex										0.69	0.86		
Female	8	53.3	12	60.0									
Male	7	46.7	8	40.0									
Metastatic site										0.53	0.59		
Lymph node	8	53.3	10	50.0									
Subcutaneous	3	20.0	8	40.0									
Lung	1	6.7	1	5.0									
Liver	1	6.7	0	0.0									
Other	2	13.3	1	5.0									
Histological parameters													
Lymph-node metastasis										0.95	0.66		
Absent	7	46.7	10	50.0									
Cortical	2	13.3	2	10.0									
Medullary	0	0.0	0	0.0									
Combined	6	40.0	8	40.0									
Extranodal extension										0.54	0.54		
Absent	9	60.0	14	70.0									
Present	6	40.0	6	30.0									
Pigmentation										0.32	0.73		
Absent	9	60.0	16	80.0									
Focal	3	20.0	3	15.0									
Heavy	3	20.0	1	5.0									
Tumour-infiltrating lymphocytes													
Absent	8	53.3	11	55.0	0.92	13	72.2	3	60.0	0.60			
Nonbrisk	2	13.3	0	0.0	0.09	1	5.6	0	0.0	0.59			
Brisk	5	33.3	9	45.0	0.49	4	22.2	2	40.0	0.42			
Predominant cytology										0.83	0.41		
Epithelioid	10	66.7	14	70.0									
Spindle	0	0.0	0	0.0									
Mixed	5	33.3	6	30.0									
Anaplasia										0.20	0.25		
Absent	5	33.3	3	15.0									
Mild	10	66.7	17	85.0									
Necrosis										0.04	0.04		
Absent	7	46.7	7	35.0	0.49	1	5.6	2	40.0	0.10			
Focal	5	33.3	9	45.0	0.49	11	61.1	1	20.0	0.10			
Extensive	3	20.0	4	20.0	1.00	6	33.3	2	40.0	0.78			

Significant at P < 0.05. UV, ultraviolet.

Clinical and histological features of BRAF mutation

In an attempt at finding morphological predictors of BRAF mutation positivity in melanoma, Viros *et al.* studied a cohort of 302 primary melanoma cases. They reported that increased upward migration and nest formation of intraepidermal melanocytes; thickening of the epidermis; sharper demarcation from the surrounding skin; and larger, rounder and more pigmented tumour cells were positive predictors of the

mutation (P < 0.0001).¹³ According to Liu *et al.*, pigmentation (OR = 3.4) and low tumour thickness (OR = 3.3) were predictors of BRAF mutation.¹⁹ In our population, no strong association was found between any of the morphological features and mutation, with the exception of epithelioid and mixed cytology (P < 0.001).

Nevertheless, the mutational rate in our population increased with other negative prognostic factors such as Clark level 4 and perineural invasion. Moreover, in

Table 5 BRAF status of patients with matched primary and metastatic melanomas.

Patient	BRAF status		BRAF status		
	Primary 1	Primary 2	Metastasis 1	Metastasis 2	Metastasis 3
1	V600E	N/A	WT	N/A	N/A
2	WT	N/A	V600E	N/A	N/A
3	WT	N/A	WT	N/A	N/A
4	V600E	V600E	WT	N/A	N/A
5	WT	N/A	WT	WT	N/A
6	WT	WT	N/A	N/A	N/A
7	N/A	N/A	V600E	V600E	N/A
8	N/A	N/A	WT	WT	WT

N/A, not applicable; WT, wild type.

contrast to Edlundh-Rose *et al.*, we found a higher rate of moderate to pronounced lymphocytic infiltration of tumours positive for the BRAF mutated gene ($P = 0.02$) in our population, and BRAF negativity was associated with the absence of tumour-infiltrating lymphocytes.^{1,9} However, because of the marginal significance of these results, we cannot conclude that more severe lesions are predictive of positive BRAF mutation.

In addition to these observations, in our cohort, the mutation was most commonly found in lymph-node metastases, but it was strongly associated only with subcutaneous metastasis. Viros *et al.* also reported frequent involvement of regional lymph-node metastasis by mutated tumours ($P < 0.0001$).¹³ In contrast to our findings, Gorden *et al.* and Pollock *et al.* denied an association between subcutaneous metastasis and BRAF mutation.^{5,23}

Ultraviolet radiation and BRAF somatic mutation in primary and metastatic melanomas

BRAF mutations are not typical UVR signature mutations as they do not involve dipyrimidine sites. In consequence, they do not seem to be the direct result of sun exposure. However, this genetic alteration was shown to occur at high frequencies on intermittently sun-exposed skin in contrast to much lower rates in non-sun-exposed locations such as mucosal melanomas, thyroid, ovarian, lung and colon cancers.¹¹ Therefore, an association between UVR and BRAF mutations has been suggested, but the precise link remains unknown.

To our knowledge, only Qi *et al.* have studied the association of UVR with BRAF mutation using direct measurements of UVR flux from three Chinese regions with different UVR exposure levels, and they did not find any effect of UVR on BRAF mutational rate. The degree of solar elastosis has been used commonly as

a histological marker of cumulative UVR dose.¹⁷ Bauer *et al.* reported lower mutational rates in regions with a higher degree of solar elastosis {39.2% in Australia [chronic sun damage (CSD) rating of 6], vs. 50.5% in Europe/USA [CSD = 4]}.²⁵ Findings consistent with this were reported by Liu *et al.* To further examine the association between sun exposure and the occurrence of BRAF mutation, previous authors have studied the mutational rate in anatomical locations with different patterns of sun exposure. Higher mutational rates have generally been detected in anatomical locations with intermittent sun exposure, but Qi *et al.* also did not find any significant association between BRAF mutational rate and anatomical location.¹⁷

In our Near East cohort, BRAF mutations occurred more frequently in regions with potentially lower UVR exposure in primary and metastatic melanomas. However, UVR was an independent negative predictor of BRAF mutation only in metastatic melanomas (OR = 0.21). Moreover, solar elastosis was not significantly associated with BRAF mutation. In our geographical region, the highest rates of BRAF mutation (37.5%) were found for the trunk and leg; however, only the trunk was a significant predictor of BRAF mutation ($P < 0.05$).

In view of our findings, an association between UVR exposure and BRAF mutation could not be confirmed. The different BRAF mutation rates between the two geographical locations studied could be a reflection of different genetic susceptibility and other factors that we could not account for in our study, such as the different UVR exposure doses to different anatomical locations being influenced by the particular traditional dress in these regions, our inability to determine the outdoor time and sun exposure habits of each individual, and our inability to track the Fitzpatrick skin type of each patient.

Conclusion

We have shown that the *BRAF* mutational rate in primary melanomas from Near East cohorts is intermediate between those of East Asian and western cohort. We have also obtained lower *BRAF* mutational rate with higher UVR exposure rates for primary and metastatic lesions. However, this may be due to factors other than potential UVR exposure. Finally, none of the studied morphological parameters was a significant positive predictor of *BRAF* mutation.

What's already known about this topic?

- With the introduction of vemurafenib, a potent inhibitor of *BRAF*, to treat melanoma, the interest in *BRAF* has recently increased.
- The *BRAF* mutation rate has been extensively studied in primary melanomas.
- However, reports of its frequency in metastatic tumours are limited, and the available data are based on homogeneous white and East Asian populations.

What does this study add?

- Our study is the first of its kind to perform extended *BRAF* testing for nine different mutations on a large series of both primary and metastatic melanomas on two Near East populations from regions with different potential annual erythemal UVR exposure.
- We also correlated the mutational status with the usual prognostic clinicopathological parameters.

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