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ORIGINAL ARTICLE

BsmI (rs1544410) and FokI (rs2228570) vitamin D receptor polymorphisms, smoking, and body mass index as risk factors of cutaneous malignant melanoma in northeast Italy

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ABSTRACT

Objective: To investigate whether vitamin D receptor gene (*VDR*) BsmI-rs1544410 and FokI-rs2228570 polymorphisms, smoking duration, and body mass index (BMI) are risk factors for cutaneous melanoma, especially metastatic melanoma.

Methods: We studied 120 cutaneous melanoma cases [68 stage I and II non-metastatic melanoma (NMetM) patients, plus 52 Stage III and IV metastatic melanoma (MetM) patients], and 120 matching healthy controls from northeast Italy. *VDR* polymorphisms were measured by restriction fragment length polymorphism analysis. Absence or presence of BsmI and FokI restriction sites was denoted by "B" and "F" or by "b" and "f," respectively.

Results: *VDR*-BsmI bb genotype was more frequent among MetM (32.7%) than among NMetM cases (13.2%), with odds ratio (OR)=3.18. Comparison of all melanoma patients *vs* healthy controls showed that the following biomarkers were at risk: ≥20 years of smoking (OR=2.43); ≥20 years of smoking combined with bb (OR=4.78), Bb+bb (OR=2.30), Ff (OR=3.04), and Ff+ff (OR=3.08); obesity (BMI>30 kg/m^2) alone (OR=3.54); and obesity combined with Bb+bb (OR=3.52), Ff (OR=4.78), and Ff+ff (OR=6.56). Comparison of MetM *vs* NMetM patients revealed that the following biomarkers were at risk: ≥20 years of smoking (OR=2.39), ≥20 years of smoking combined with bb (OR=5.13), Bb+bb (OR=3.07), and Ff+ff (OR=2.66); and obesity combined with Bb+bb (OR=5.27), Ff (OR=6.28), and Ff+ff (OR=9.18). Triple combination of ≥20 years of smoking, obesity, and Bb+bb yielded OR=9.65 for melanoma patients *vs* healthy controls and OR=12.2 for MetM *vs*. NMetM patients.

Conclusions: Risk factors for cutaneous MetM include two *VDR* polymorphisms combined with smoking duration and obesity. Results suggest gene-environment implications in melanoma susceptibility and severity. Future studies in larger cohorts and in subjects with different genetic background are warranted to extend our findings.

KEYWORDS Vitamin D receptor; VDR polymorphism; cutaneous melanoma; metastatic melanoma; smoking; body mass index; obesity; skin cancer

Introduction

Melanoma continually presents increased incidence in all developed countries, particularly affecting fair-skinned individuals[1-3]. Malignant melanoma more frequently occurs in northern than in southern European countries[3]. Melanoma more frequently affects both sexes in Switzerland (European age standardized incidence rate 25.8/100,000/year) and Slovenia (20.6/100,000/year) than in Italy (13.4/100,000/year)[4]. Recent data in Italy[5] indicated a more than doubled prevalence of melanoma in northern than southern Italy, with central Italy presenting an intermediate value. Specifically, high incidence rates were recorded in Friuli-Venezia Giulia (FVG) region (19.6/100,000/year in men; 16.4/100,000/year in women) in northeast Italy[6]. This finding implies necessity for conducting geographically detailed studies regarding melanoma risk factors[7]. In the present study, we focused on inhabitants of the FVG region.

Critical environmental risk factors for melanoma include exposure to ultraviolet (UV) radiation, especially intermittent sun exposure and sunburns[8,9]. However, chronic and continuous UV ray exposure may yield protective effects[9,10] at least in part by activating synthesis of vitamin D, whose action is mediated by nuclear vitamin D receptor (VDR). Vitamin D-activated VDR may in turn up- or down-regulate several hundreds of genes by binding to vitamin D

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responsive elements (VDREs), thus affecting several biological activities, such as calcium metabolism, immunity, detoxification, oxidative stress, cell proliferation, and differentiation[9-12]. Increasing evidence showed that vitamin D reduces risk of numerous types of cancer[12]. Thus, vitamin D endocrine system in studies concerning melanoma gained increasing attention[10,13,14]. Current studies and meta-analyses evaluated the role of the VDR gene (*VDR*) polymorphisms[12-22]. Nonetheless, *VDR* polymorphisms' roles still require further study[12,14,21].

The role of smoking in melanoma piqued interest of researchers[23,24]. Smoking is considered a risk factor for malignancies[25]. Paradoxically, several studies discovered inverse associations between smoking exposure and melanoma after controlling for potential confounding variables[24,26,27]. However, such protective effects are weak or insignificant[28,29]. Other studies did not confirm such association[30,31] or demonstrated tendencies toward smoking-related increased risks[10,32]. Thus, pathophysiological pathways underlying the relationship of smoking and melanoma currently poses a challenge in melanoma research[23,24].

Some studies on melanoma aimed to determine the role of body mass index (BMI) in occurrence of the disease[9,10,33-35]. However, limited research discusses combination of this biomarker with genetic traits.

Development in understanding of melanoma risk factors, genomics, and molecular pathogenesis may drive advances in precision medicine applied to melanoma[2,13,14].

Human *VDR* gene is located in chromosome 12q12-q14 and comprises 11 exons and 11 introns[18-22]. Most clinical studies that explored association of *VDR* polymorphisms with diseases[12,15,18,22] focused on two VDR single-nucleotide polymorphisms (SNPs), namely, BsmI-rs1544410 G>A located in intron 8 and FokI-rs2228570 C>T located in exon 2. These two polymorphisms show no linkage disequilibrium (LD)[18,22].

We explored *VDR* BsmI-rs1544410 and FokI-rs2228570 SNPs separately, and their association with lifestyle factors, particularly smoking duration and BMI of patients with cutaneous malignant melanomas, specifically those with metastatic melanoma (MetM) *vs.* non-metastatic melanoma (NMetM) and *vs.* healthy controls.

Patients and methods

Population

Enrollment and clinical visits of all study participants were performed at Dermatology Clinic, University Hospital of Udine. Diagnostic procedures were carried out according to routine protocols. The Udine Institutional Ethical Committee approved the study protocol, which was conducted according to the Declaration of Helsinki. All participants were alive during enrollment in the study and signed a written informed consent.

Using a case-control design, the study consecutively enrolled 120 (65 males and 55 females, age range of 31–84 years) unrelated patients (hospitalized or outpatients) with documented cutaneous melanoma diagnosis and 120 (65 males and 55 females, age range of 31–84 years) asymptomatic healthy controls, which were matched for gender, ancestry, and age with melanoma cases. Inclusion criteria for both melanoma cases and healthy controls were as follows: resident in FVG region, at least two grandparents born in FVG region (or Austro-Hungarian territory before World War I), and two grandparents, at the most, with central or southern Italian ancestry. Exclusion criteria for controls included the following: any kind of lifelong malignant or benign tumor, first-grade relatives with history of melanoma, and major chronic diseases, such as autoimmune diseases, type 1 diabetes, and thyroid diseases.

Melanoma was diagnosed using immunohistological findings obtained after surgical excision of nevi with clinical and dermoscopic characteristics suggesting presence of malignancy. Classification of melanoma stages was performed by clinical/histological/radiological findings, as described in final version of 2009 AJCC[36]. Inclusion criteria for case patients comprised cutaneous melanomas that were more severe than *in situ* only and with a Clark-grade invasion over I. For patients with multiple melanomas, the major melanoma characteristics were accounted for in study analyses according to histological assessment of major primary tumor (T) grading.

Each participant answered a questionnaire, which was used to collect data on demographic characteristics, medical and family history of melanoma, smoking habits, alimentary habits, and history of sunburns. Phototype was assessed by Fitzpatrick criteria[37]. BMI was determined by weight (kg) divided by squared height (m^2); BMI>30 kg/m^2 was considered as an indicator of obesity.

Genetic analysis of *VDR* polymorphisms

VDR-BsmI G>A and *VDR*-FokI C>T polymorphisms were determined, as described in Refs.[38,39], after extraction of genomic DNA from ethylenediaminetetraacetic-acid-treated venous blood samples[40]. Genotypes were designated according to absence/presence of the BsmI or FokI enzyme

restriction site by a capital letter B allele, or F allele for absence, and by a lowercase letter b allele, or f allele for presence, respectively[41]. FokI and BsmI polymorphisms of *VDR* were studied using previously tested primers[38-40], which were used to amplify appropriate DNA fragments. The following primers were specifically used: FokI-forward (5′-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′) and FokI-reverse (5′-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′); BsmI-forward (5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′) and BsmI-reverse (5′-AAC CAG CGG GAA GAG GTC AAG GG-3′) primers. FokI enzyme (Euroclone, Milano, Italy) digestion of amplified 265 bp DNA fragment resulted in two 196 and 69 bp fragments in the presence of f allele[40]. To analyze BsmI polymorphism, the resulting amplified 825 bp fragment was digested with BsmI restriction enzyme (Euroclone, Milano, Italy), generating two fragments of 650 and 175 bp in the presence of b allele[39].

Statistical analysis

Continuous variables were expressed as mean ± standard deviation, and Mann-Whitney *U* test was performed for comparison. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for categorical variables, and *P* values for two-sided Pearson's Chi-squared or Fisher's exact test were reported as appropriate. Logistic regression was used to evaluate effects of confounders by obtaining adjusted ORs and CIs. Five different combinations of confounders were tested. Adjusted analysis included conventional risk factors: (1) gender and age; (2) gender, age, phototype 1+2, total number of body nevi>50, and number of lifelong sunburns>10. To compare MetM and NMetM, adjusted analyses included indicators that resulted in risk of metastasis development: (3) trunk location, Breslow's thickness, ulceration, mitosis>1, absence of tumor-infiltrating lymphocytes (TILs), and epithelioid variant; (4) ≥20 years of smoking; and (5) BMI>30 kg/m^2 (i.e., obesity). Adjusted analysis of type 3 confounders involved factors associated (according to our findings) with ≥20 years of smoking. These factors included TIL absence, ulceration and obesity. Thus, to avoid overcorrection, combined categorical variables, including smoking and obesity, were not adjusted for type 3 confounders.

Tests for deviations from Hardy-Weinberg equilibrium (HWE) were separately performed using chi-square distribution for each SNP[39,40]. LD between SNPs was determined as described by Colombini et al.[39]

A two-sided value of $P<0.05$ was considered significant, and $P\leq0.10$ indicates tendency to be significant. Statistical software SPSS for Windows (SPSS Inc., Chicago, IL, USA) was used.

Results

All 240 (120 cutaneous melanoma patients + 120 healthy controls) study subjects were Italian white residents in the FVG region.

Primary clinical characteristics of melanoma patients

As reported in **Table 1**, we examined in detail differences between MetM and NMetM patients to also identify appropriate variables to be included as confounders in subsequent multivariate analyses. Frequency of young (<40 years old) or old (≥60 years old) melanoma patients at study enrolment did not differ between MetM and NMetM groups (mean age comparisons reported in **Table 2**). Mean age at melanoma diagnosis reached 53.1±13.26 years. Mean time for melanoma diagnosis totaled 6.5±3.58 years and did not differ between MetM and NMetM patients.

The majority of 68 NMetM patients were in stage I (70.6%), whereas the majority of 52 MetM patients were in stage III (65.4%). Location in the trunk (OR=0.35) and superficial spreading (OR=0.31) showed protective effects for MetM patients *vs*. NMetM patients. Mean Breslow's thickness doubled in MetM cases *vs*. NMetM cases (2.8±1.74 *vs*. 1.4±1.34 mm, $P<0.001$). Specifically, a Breslow's thickness ≤0.75 mm had protective effects (OR=0.06), whereas thickness ≥4.01 mm was risky (OR=9.90) for MetM *vs*. NMetM cases. Some biomarkers were more frequently observed in MetM than in NMetM patients. These biomarkers included Clark IV invasion (OR=4.38), ulceration (OR=3.79), mitosis >1 (OR=3.77), TIL absence (OR=2.20), and epithelioid variant (OR=2.98).

Obesity and smoking history

By comparing obese and non-obese melanoma patients, we observed that non-brisk TIL cases were less frequent in obese (1/16, 6.25%) than in non-obese (40/104, 38.5%) patients, with OR=0.11, 95% CI=0.01–0.84, and $P=0.011$. By contrast, TIL absence was more frequent in obese (10/16, 62.5%) than in non-obese (31/104, 29.8%) melanoma patients, resulting in OR=3.92, 95% CI=1.31–11.7, and $P=0.010$.

Similar findings were observed by comparing melanoma patients who smoked ≥20 years *vs*. the remaining melanoma patients. Frequency of non-brisk TIL cases was lower in ≥20-year smokers (7/36, 19.4%) than other melanoma patients (34/84, 40.5%), yielding OR=0.35, 95% CI=0.14–0.90, and $P=0.026$. By contrast, TIL absence was more frequent in ≥20-

Table 1 Clinical characteristics of 120 consecutively enrolled melanoma patients and comparison between the two subgroups of 52 MetM and 68 NMetM patients

Characteristics	All melanoma patients (*n*=120)	MetM (*n*=52)	NMetM (*n*=68)	OR (CI) (MetM *vs*. NMetM)	*P* (MetM *vs*. NMetM)
Age, years, *n* (%)					
<40	9 (7.5)	3 (5.8)	6 (8.8)	0.63 (0.15–2.66)	0.730
≥ 60	60 (50.0)	27 (51.9)	33 (48.5)	1.14 (0.56–2.36)	0.713
Age at melanoma diagnosis (years, mean±SD)	53.1±13.26	53.9±13.01	52.5±13.51	–	0.569[a]
Time from melanoma diagnosis (years, mean±SD)	6.5±3.58	6.3±4.13	6.6±3.08	–	0.344[a]
Stage, *n* (%)					
I	48 (40.0)	0 (–)	48 (70.6)	–[b]	–[b]
II	20 (16.7)	0 (–)	20 (29.4)	–[b]	–[b]
III	34 (28.3)	34 (65.4)	0 (–)	–[b]	–[b]
IV	18 (15.0)	18 (34.6)	0 (–)	–[b]	–[b]
Trunk, *n* (%)	68 (56.7)	22 (42.3)	46 (67.6)	**0.35 (0.17–0.74)**	**0.006**
Upper limb, *n* (%)	8 (6.7)	2 (3.8)	6 (8.8)	0.41 (0.08–2.14)	0.463
Lower limb, *n* (%)	26 (21.7)	15 (28.8)	11 (16.2)	2.10 (0.87–5.07)	0.095^
Hands/feet, *n* (%)	8 (6.7)	6 (11.5)	2 (2.9)	4.30 (0.83–22.3)	0.076^
Head/neck, *n* (%)	10 (8.3)	7 (13.5)	3 (4.4)	3.37 (0.83–13.7)	0.099^
Superficial spreading, *n* (%)	56 (46.7)	16 (30.8)	40 (58.8)	**0.31 (0.14–0.67)**	**0.002**
Nodular, *n* (%)	47 (39.2)	25 (48.1)	22 (32.4)	1.94 (0.92–4.07)	0.080^
Acral lentiginous, *n* (%)	5 (4.2)	4 (7.7)	1 (1.5)	5.58 (0.60–51.5)	0.165
Lentigo maligna, *n* (%)	2 (1.7)	1 (1.9)	1 (1.5)	1.31 (0.08–21.5)	1.000
Spitzoide, *n* (%)	5 (4.2)	2 (3.8)	3 (4.4)	0.87 (0.14–5.38)	1.000
Others, *n* (%)	8 (6.7)	6 (11.5)	2 (2.9)	4.30 (0.83–22.3)	0.076^
Breslow thickness (mm, mean±SD)	2.0±1.66	**2.8±1.74**	**1.4±1.34**	–	**<0.001[a]**
Breslow thickness ≤0.75 mm, *n* (%)	28 (23.3)	2 (3.8)	26 (38.2)	**0.06 (0.01–0.29)**	**<0.001**
Breslow thickness ≥4.01 mm, *n* (%)	14 (11.7)	12 (23.1)	2 (2.9)	**9.90 (2.11–46.5)**	**0.001**
Clark II, *n* (%)	29 (24.2)	4 (7.7)	25 (36.8)	**0.14 (0.05–0.44)**	**<0.001**
Clark III, *n* (%)	20 (16.7)	4 (7.7)	16 (23.5)	**0.27 (0.08–0.87)**	**0.021**
Clark IV, *n* (%)	64 (53.3)	38 (73.1)	26 (38.2)	**4.38 (2.00–9.60)**	**<0.001**
Clark V, *n* (%)	5 (4.2)	4 (7.7)	1 (1.5)	5.58 (0.60–51.5)	0.165
Ulceration, *n* (%)	48 (40.0)	30 (57.7)	18 (26.5)	**3.79 (1.75–8.18)**	**0.001**
Mitosis >1, *n* (%)	81 (67.5)	43 (82.7)	38 (55.9)	**3.77 (1.59–8.94)**	**0.002**
Regression, *n* (%)	16 (13.3)	4 (7.7)	12 (17.6)	0.39 (0.12–1.28)	0.112
Brisk positive TILs[c], *n* (%)	37 (30.8)	12 (23.1)	25 (36.8)	0.52 (0.23–1.16)	0.108
Non-brisk TILs[c], *n* (%)	41 (34.2)	16 (30.8)	25 (36.8)	0.76 (0.35–1.65)	0.493

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Characteristics	All melanoma patients (*n*=120)	MetM (*n*=52)	NMetM (*n*=68)	OR (CI) (MetM *vs*. NMetM)	*P* (MetM *vs*. NMetM)
TILs[c] absence, *n* (%)	41 (34.2)	23 (44.2)	18 (26.5)	**2.20 (1.02–4.75)**	**0.042**
Microsatellitosis, *n* (%)	4 (3.3)	3 (5.8)	1 (1.5)	4.10 (0.41–40.6)	0.315
Epithelioid variant, *n* (%)	30 (25.0)	19 (36.5)	11 (16.2)	**2.98 (1.27–7.03)**	**0.011**
Fusate variant, *n* (%)	12 (10.0)	7 (13.5)	5 (7.4)	1.96 (0.58–6.57)	0.269
Small cell variant, *n* (%)	2 (1.7)	0 (–)	2 (2.9)	–[b]	–[b]
More than 1 melanoma, *n* (%)	18 (15.0)	9 (17.3)	9 (13.2)	1.37 (0.50–3.74)	0.536
Additional non-melanoma skin cancer, *n* (%)	18 (15.0)	7 (13.5)	11 (16.2)	0.81 (0.29–2.25)	0.680
Additional non-skin cancer, *n* (%)	23 (19.2)	11 (21.2)	12 (17.6)	1.25 (0.50–3.12)	0.629
Melanoma familiarity	17 (14.2)	7 (13.5)	10 (14.7)	0.90 (0.32–2.57)	0.846

[a]Two-tailed Mann-Whitney *U*-test.[b]OR uncountable because one or two of the compared groups had zero subject. [c]TILs, tumor infiltrating lymphocytes. Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

Table 2 Comparison of demographic characteristics of 120 melanoma patients and 120 healthy controls and comparison between the two subgroups of 52 MetM and 68 NMetM patients

Characteristics	All melanoma cases *n*=120	Healthy controls *n*=120	OR (CI) (melanomas *vs*. healthy controls)	*P* (melanomas *vs*. healthy controls)	MetM *n*=52	NMetM *n*=68	OR (CI) (MetM *vs*. NMetM)	*P* (MetM *vs*. NMetM)
Age, years, mean±SD	59.1±12.8	56.8±11.8	–	0.110[a]	60.2±12.1	58.3±13.4	–	0.503[a]
Age <50 years, *n* (%)	34 (28.3)	37 (30.8)	0.89 (0.51–1.54)	0.671	11 (21.1)	23 (33.8)	0.52 (0.23–1.21)	0.127
Males, *n* (%)	65 (54.2)	65 (54.2)	1.00 (0.60–1.66)	1.000	32 (61.5)	33 (48.5)	1.70 (0.81–3.53)	0.156
BMI, kg/m^2, mean±SD	**25.7±3.89**	**24.4±3.35**	–	**0.010[a]**	26.1±3.81	25.4±3.94	–	0.392[a]
BMI >25.0 kg/m^2, *n* (%)	63 (52.5)	52 (43.3)	1.44 (0.87–2.40)	0.155	30 (57.7)	33 (48.5)	1.45 (0.70–2.99)	0.319
BMI >30.0 kg/m^2, *n* (%)	16 (13.3)	5 (4.2)	**3.54 (1.25–10.0)**	**0.012**	9 (17.3)	7 (10.3)	1.82 (0.63–5.27)	0.263
Born in FVG region, *n* (%)	99 (82.5)	100 (83.3)	0.94 (0.48–1.85)	0.864	40 (76.9)	59 (86.8)	0.51 (0.20–1.32)	0.160
All 4 grand-parents born in FVG region, *n* (%)	85 (70.8)	83 (69.2)	1.08 (0.62–1.88)	0.778	35 (67.3)	50 (73.5)	0.74 (0.34–1.63)	0.457
Elementary school (5 study years), *n* (%)	17 (14.2)	8 (6.7)	2.31 (0.96–5.58)	0.057^	10 (19.2)	7 (10.3)	2.07 (0.73–5.89)	0.164
Low high-school (8 study years), *n* (%)	34 (28.3)	21 (17.5)	**1.86 (1.01–3.45)**	**0.046**	18 (34.6)	16 (23.5)	1.72 (0.77–3.83)	0.182
High-school (13 study years), *n* (%)	54 (45.0)	42 (35.0)	1.52 (0.90–2.55)	0.114	21 (40.4)	33 (48.5)	0.72 (0.35–1.49)	0.374
University level (laurea and/or master and/or PhD), *n* (%)	15 (12.5)	49 (40.8)	**0.21 (0.11–0.40)**	**<0.001**	3 (5.8)	12 (17.6)	0.29 (0.08–1.07)	0.051^

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Characteristics	All melanoma cases *n*=120	Healthy controls *n*=120	OR (CI) (melanomas *vs*. healthy controls)	*P* (melanomas *vs*. healthy controls)	MetM *n*=52	NMetM *n*=68	OR (CI) (MetM *vs*. NMetM)	*P* (MetM *vs*. NMetM)
Phototype 1 or 2, *n* (%)	71 (59.2)	31 (25.8)	**4.16 (2.41–7.19)**	**<0.001**	34 (65.4)	37 (54.4)	1.58 (0.75–3.33)	0.226
Total Body Nevi >50, *n* (%)	63 (52.5)	41 (34.2)	**2.13 (1.27–3.58)**	**0.004**	28 (53.8)	35 (51.5)	1.10 (0.53–2.27)	0.796
Easy tanner, *n* (%)	4 (3.3)	22 (18.3)	**0.15 (0.05–0.46)**	**<0.001**	0 (–)	4 (5.9)	–[b]	–[b]
Medium tanner, *n* (%)	50 (41.7)	71 (59.2)	**0.49 (0.29–0.82)**	**0.007**	22 (42.3)	28 (41.2)	1.05 (0.50–2.18)	0.901
Low tanner, *n* (%)	58 (48.3)	26 (21.7)	**3.38 (1.93–5.94)**	**<0.001**	27 (51.9)	31 (45.6)	1.29 (0.62–2.66)	0.491
No tanner, *n* (%)	8 (6.7)	2 (1.7)	4.21 (0.88–20.3)	0.053^	3 (5.8)	5 (7.4)	0.77 (0.18–3.39)	1.000
Sunburns lifelong ≤5, *n* (%)	37 (30.8)	58 (48.3)	**0.48 (0.28–0.81)**	**0.006**	14 (26.9)	23 (33.8)	0.72 (0.33–1.59)	0.417
Sunburns lifelong 6–10, *n* (%)	21 (17.5)	28 (23.3)	0.70 (0.37–1.31)	0.262	12 (23.1)	9 (13.2)	1.97 (0.76–5.10)	0.160
Sunburns lifelong >10, *n* (%)	51 (42.5)	27 (22.5)	**2.55 (1.45–4.46)**	**0.001**	21 (40.4)	30 (44.1)	0.86 (0.41–1.78)	0.682
Present-smoker, *n* (%)	13 (10.8)	20 (16.7)	0.61 (0.29–1.29)	0.189	5 (9.6)	8 (11.8)	0.80 (0.24–2.60)	0.707
≥20 years smoking among present-smokers, *n* (%)	11 (84.6)	12 (60.0)	3.67 (0.64–21.1)	0.245	4 (80.0)	7 (87.5)	0.57 (0.03–11.8)	1.000
Years of smoking among present-smokers, mean±SD	27.7±13.7	23.4±13.5	–	0.386[a]	25.4±7.96	29.1±16.7	–	0.558[a]
N. cigarettes/day among present-smokers, mean±SD	14.4±9.99	9.83±6.26	–	0.234[a]	13.2±6.98	15.1±11.9	–	0.941[a]
Past-smoker, *n* (%)	46 (38.3)	28 (23.3)	**2.04 (1.17–3.58)**	**0.012**	23 (44.2)	23 (33.8)	1.55 (0.74–3.26)	0.245
≥20 years smoking among past-smokers, *n* (%)	25 (54.3)	6 (21.4)	**4.36 (1.49–12.8)**	**0.005**	17 (73.9)	8 (34.8)	**5.31 (1.50–18.8)**	**0.008**
Years of smoking among past-smokers, mean±SD	20.5±12.9	17.4±13.2	–	0.237[a]	**24.5±13.9**	**16.4±10.6**	–	**0.039[a]**
N. cigarettes/day among past-smokers, mean±SD	14.9±10.5	16.0±13.2	–	0.964[a]	13.8±9.85	16.0±11.3	–	0.424[a]
Years quitting smoking among past-smokers, mean±SD	20.2±12.3	20.6±14.5	–	0.978[a]	20.0±13.0	20.3±11.9	–	0.895[a]
Quitted smoking before first melanoma diagnosis among past-smokers, *n* (%)	39 (84.8)	–	–	–	19 (82.6)	20 (87.0)	0.71 (0.14–3.61)	1.000
Years of quitted smoking before first melanoma diagnosis, mean±SD	16.2±10.1	–	–	–	15.8±10.1	16.6±10.3	–	0.899
Ever-smoker, *n* (%)	59 (49.2)	48 (40.0)	1.45 (0.87–2.42)	0.153	28 (53.8)	31 (45.6)	1.39 (0.67–2.87)	0.370

Continued

Continued

Characteristics	All melanoma cases *n*=120	Healthy controls *n*=120	OR (CI) (melanomas *vs.* healthy controls)	*P* (melanomas *vs.* healthy controls)	MetM *n*=52	NMetM *n*=68	OR (CI) (MetM *vs.* NMetM)	*P* (MetM *vs.* NMetM)
≥20 years smoking among ever-smokers, n (%)	36 (61.0)	18 (37.5)	**2.61 (1.19–5.72)**	**0.016**	21 (75.0)	15 (48.4)	**3.20 (1.06–9.69)**	**0.036**
Years of smoking among ever-smokers, mean±SD	22.0±13.3	19.9±13.5	–	0.298[a]	24.7±12.9	19.7±13.4	–	0.086^[a]
N. cigarettes/day, among ever-smokers, mean±SD	14.8±10.3	13.4±11.2	–	0.335[a]	13.7±9.29	15.8±11.2	–	0.451[a]
≥20 years ever-smokers among all subjects, n (%)	36 (30.0)	18 (15.0)	**2.43 (1.29–4.58)**	**0.005**	21 (40.4)	15 (22.1)	**2.39 (1.08–5.31)**	**0.030**
Coffee drinker daily, n (%)	110 (91.7)	112 (93.3)	0.79 (0.30–2.06)	0.624	46 (88.5)	64 (94.1)	0.48 (0.13–1.79)	0.327
Coffee cups/day >3, n (%)	20 (16.7)	17 (14.2)	1.21 (0.60–2.45)	0.592	11 (21.1)	9 (13.2)	1.76 (0.67–4.62)	0.249

[a]Two-tailed Mann-Whitney *U*-test. [b]OR uncountable because one of the compared group had zero subject. Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

year smokers (17/36, 47.2%) than in other melanoma patients (24/84, 28.6%), with OR=2.24, 95% CI=1.00–5.02, and *P*=0.048. By comparing ≥20-year smokers with the remaining melanoma patients, we detected significant findings for males (OR=4.45, 95% CI=1.81–10.9, *P*=0.001), stage III melanoma (OR=2.44, 95% CI=1.06–5.64, *P*=0.034), and ulceration (OR=2.50, 95% CI=1.12–5.56, *P*=0.023).

Comparison of demographic, behavioral, and environmental variables (Table 2)

Melanoma patients yielded higher mean BMI than healthy controls (*P*=0.010), and the number of obese subjects was over threefold higher (OR=3.54) among melanoma patients than among healthy controls.

Melanoma patients more frequently presented phototype 1+2 (OR=4.16), total number of body nevi>50 (OR=2.13), lifelong sunburns>10 (OR=2.55), and were more frequently low tanners (OR=3.38) than healthy controls. MetM patients did not differ from NMetM patients in terms of these characteristics.

Past smokers were twofold more frequent among melanoma patients than healthy controls. Among past smokers smoking for ≥20 years was considerably more frequent in melanoma patients (OR=4.36) than healthy controls and in MetM (OR=5.31) than NMetM patients. Past smokers with MetM showed higher average number of smoking years than NMetM patients (24.5±13.9 *vs.* 16.4±10.6 years; *P*=0.039). Among past smokers, melanoma patients quitted smoking for an average of 16.2±10.1 years before melanoma diagnosis, and differences were not observed between MetM and NMetM patients.

Twenty or more years of smoking among lifelong smokers and among all study subjects was a risk factor for melanoma patients *vs.* healthy controls (OR=2.61 and OR=2.43, respectively) and for MetM *vs.* NMetM patients (OR=3.20 and OR=2.39, respectively).

The majority of melanoma patients and healthy controls were daily coffee drinkers; no difference was noted among groups even when considering those who consumed over three cups of coffee per day.

Unadjusted comparisons of *VDR*-BsmI and *VDR*-FokI genotypes alone or combined with smoking and obesity (Table 3)

VDR-BsmI and *VDR*-FokI genotypes were in HWE in healthy controls and in melanoma patients. As expected, the two SNPs were not in LD.

Homozygous bb genotype was more frequent among MetM than among NMetM patients (OR=3.18). Intriguingly, bb frequency was lower in NMetM patients than in healthy controls (OR=0.40). Genotype bb combined with ≥20 years of smoking was more frequent among all melanoma patients than healthy controls (OR=4.78), in MetM than NMetM patients (OR=5.13), and in MetM patients than healthy controls (OR=9.18). The same profile was observed for Bb+bb (b allele carriers) plus ≥20 years of smoking. Carriers

Table 3 *VDR*-BsmI and *VDR*-FokI genotypes alone or combined with smoking duration and obesity compared between 120 melanoma cases and 120 healthy controls.

Single or combined variable	Melanoma cases (*n*=120), *n* (%)	Healthy controls (*n*=120), *n* (%)	OR (CI), *P* (Melanomas *vs*. healthy controls)	MetM (*n*=52), *n* (%)	NMetM (*n*=68), *n* (%)	OR (CI), *P* (MetM *vs*. NMetM)	OR (CI), *P* (MetM *vs*. healthy controls)	OR (CI), *P* (NMetM *vs*. healthy controls)
BB	30 (25.0)	31 (25.8)	0.96 (0.53–1.71), 0.882	11 (21.2)	19 (27.9)	0.69 (0.30–1.62), 0.395	0.77 (0.35–1.68), 0.512	1.11 (0.57–2.17), 0.753
Bb	64 (53.3)	56 (46.7)	1.31 (0.79–2.17), 0.302	24 (46.2)	40 (58.8)	0.60 (0.29–1.24), 0.168	0.98 (0.51–1.88), 0.951	1.63 (0.89–2.98), 0.109
bb	26 (21.7)	33 (27.5)	0.73 (0.40–1.32), 0.294	17 (32.7)	9 (13.2)	**3.18 (1.28–7.91), 0.010**	1.28 (0.63–2.59), 0.491	**0.40 (0.18–0.90), 0.024**
BB+Bb (B allele)	94 (78.3)	87 (72.5)	1.37 (0.76–2.48), 0.294	35 (67.3)	59 (86.8)	**0.31 (0.13–0.78), 0.010**	0.78 (0.39–1.58), 0.491	**2.49 (1.11–5.58), 0.024**
Bb+bb (b allele)	90 (75.0)	89 (74.2)	1.04 (0.58–1.87), 0.882	41 (78.8)	49 (72.1)	1.44 (0.62–3.38), 0.395	1.30 (0.59–2.83), 0.512	0.90 (0.46–1.75), 0.753
bb plus ≥20 years ever-smoking	9 (7.5)	2 (1.7)	**4.78 (1.01–22.6), 0.031**	7 (13.5)	2 (2.9)	**5.13 (1.02–25.8), 0.039**	**9.18 (1.84–45.8), 0.004**	1.79 (0.25–13.0), 0.621
Bb+bb plus ≥20 years ever-smoking	28 (23.3)	14 (11.7)	**2.30 (1.14–4.64), 0.017**	18 (34.6)	10 (14.7)	**3.07 (1.27–7.41), 0.011**	**4.01 (1.80–8.90), <0.001**	1.30 (0.55–3.12), 0.549
bb plus BMI >30 kg/m^2	4 (3.3)	0 (–)	–[a]	3 (5.8)	1 (1.5)	4.10 (0.41–40.6), 0.315	–[a]	–[a]
Bb+bb plus BMI >30 kg/m^2	13 (10.8)	4 (3.3)	**3.52 (1.11–11.1), 0.024**	8 (15.4)	5 (7.4)	2.29 (0.70–7.47), 0.161	**5.27 (1.51–18.4), 0.008**	2.30 (0.60–8.88), 0.288
Bb+bb plus ≥20 years ever-smoking, and plus BMI >30 kg/m^2	9 (7.5)	1 (0.8)	**9.65 (1.20–77.4), 0.010**	8 (15.4)	1 (1.5)	**12.2 (1.47–101), 0.010**	**21.6 (2.63–178), <0.001**	1.78 (0.11–28.9), 1.00
FF	47 (39.2)	54 (45.0)	0.79 (0.47–1.31), 0.360	17 (32.7)	30 (44.1)	0.61 (0.29–1.30), 0.204	0.59 (0.30–1.17), 0.132	0.96 (0.53–1.76), 0.907
Ff	60 (50.0)	50 (41.7)	1.40 (0.84–2.33), 0.195	29 (55.8)	31 (45.6)	1.50 (0.73–3.11), 0.269	1.76 (0.91–3.40), 0.088^	1.17 (0.64–2.14), 0.602
ff	13 (10.8)	16 (13.3)	0.79 (0.36–1.72), 0.552	6 (11.5)	7 (10.3)	1.14 (0.36–3.61), 0.828	0.85 (0.31–2.31), 0.746	0.75 (0.29–1.91), 0.541
FF+ff (F allele)	107 (89.2)	104 (86.7)	1.27 (0.58–2.76), 0.552	46 (88.5)	61 (89.7)	0.88 (0.28–2.79), 0.828	1.18 (0.43–3.21), 0.746	1.34 (0.52–3.44), 0.541
Ff+ff (f allele)	73 (60.8)	66 (55.0)	1.27 (0.76–2.12), 0.360	35 (67.3)	38 (55.9)	1.62 (0.77–3.45), 0.204	1.68 (0.85–3.33), 0.132	1.04 (0.57–1.89), 0.907

Continued

Continued

Single or combined variable	Melanoma cases (*n*=120), *n* (%)	Healthy controls (*n*=120), *n* (%)	OR (CI), *P* (Melanomas *vs.* healthy controls)	MetM (*n*=52), *n* (%)	NMetM (*n*=68), *n* (%)	OR (CI), *P* (MetM *vs.* NMetM)	OR (CI), *P* (MetM *vs.* healthy controls)	OR (CI), *P* (NMetM *vs.* healthy controls)
Ff plus ≥20 years ever-smoking	19 (15.8)	7 (5.8)	**3.04 (1.23–7.52), 0.013**	12 (23.1)	7 (10.3)	2.61 (0.95–7.21), 0.057^	**4.84 (1.78–13.2), 0.001**	1.85 (0.62–5.53), 0.263
Ff+ff plus ≥20 years ever-smoking	24 (20.0)	9 (7.5)	**3.08 (1.37–6.95), 0.005**	15 (28.8)	9 (13.2)	**2.66 (1.06–6.69), 0.034**	**5.00 (2.02–12.4), <0.001**	1.88 (0.71–4.99), 0.199
Ff plus BMI >30 kg/m^2	9 (7.5)	2 (1.7)	**4.78 (1.01–22.6), 0.031**	5 (9.6)	4 (5.9)	1.70 (0.43–6.68), 0.499	**6.28 (1.18–33.5), 0.027**	3.69 (0.66–20.7), 0.191
Ff+ff plus BMI >30 kg/m^2	12 (10.0)	2 (1.7)	**6.56 (1.43–30.0), 0.006**	7 (13.5)	5 (7.4)	1.96 (0.58–6.57), 0.269	**9.18 (1.84–45.8), 0.004**	4.68 (0.88–24.8), 0.101
Ff+ff plus ≥20 years ever-smoking, and plus BMI >30 kg/m^2	6 (5.0)	0 (–)	–[a]	6 (11.5)	0 (–)	–[a]	–[a]	–[a]

[a]OR uncountable because one or two of the compared groups had zero subject. Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

of b allele (Bb+bb) who were obese showed increased risk for all melanomas (OR=3.52 for melanoma patients *vs.* healthy controls) and MetM (OR=5.27 for MetM *vs.* healthy controls). Notably, the combination of three parameters, i.e., Bb+bb genotype plus ≥20 years of smoking plus obesity yielded high ORs for all melanoma patients *vs.* healthy controls (OR=9.65), MetM *vs.* NMetM patients (OR=12.2), and MetM patients *vs.* healthy controls (OR=21.6).

As shown in **Table 3**, *VDR*-FokI genotype FF, Ff, and ff frequencies did not differ among groups. However, heterozygous Ff had a tendency to be more frequent among MetM patients than healthy controls (OR=1.76; *P*=0.088). Notably, Ff genotype combined with ≥20 years of smoking acted as risk factor for all melanoma patients (OR=3.04 for melanoma patients *vs.* healthy controls) and MetM patients (OR=4.84 for MetM patients *vs.* healthy controls). Carriers of f allele (i.e., Ff+ff) combined with ≥20 years of smoking posed risk for all melanoma patients (OR=3.08 for melanoma patients *vs.* healthy controls) and for MetM (OR=5.00 for MetM patients *vs.* healthy controls, and OR=2.66 for MetM *vs.* NMetM patients). Ff genotype combined with obesity exhibited OR=4.78 for all melanoma patients *vs.* healthy controls and OR=6.28 for MetM patients *vs.* healthy controls. Finally, obese carriers of Ff+ff presented an increased risk for all melanomas (OR=6.56 for all melanoma patients *vs.* healthy controls) and for MetM (OR=9.18 for MetM patients *vs.* healthy controls). Notably, only 6 out of 240 study subjects showed the triple combination of Ff+ff genotype, ≥20 years of smoking, and obesity, and they were all MetM patients.

Comparisons of *VDR*-BsmI and *VDR*-FokI genotypes, smoking, and obesity alone or their combinations (Table 4)

As shown in **Table 4**, by comparing all 120 melanoma cases *vs.* 120 healthy controls, four variables including the parameter ≥20 years of ever smoking among all subjects were significant after multivariate analysis of type 1 confounders (including gender and age): ≥20 years of smoking alone (OR=2.19), or plus Bb+bb (OR=2.08), plus Ff (OR=2.77), and plus Ff+ff (OR=2.86) genotype. However, all those differences became not significant adding more confounding factors by analysis of type 2 confounders. Multivariate analysis of type 2 confounders revealed that five variables, including obesity, were all risk factors for melanoma patients *vs.* healthy controls, and they were as follows: BMI>30 kg/m^2 alone (OR=5.28), or plus Bb+bb (OR=4.35), plus Ff (OR=6.91), and plus Ff+ff (OR=8.89) genotype, and triple combination of Bb+bb, ≥20 years of smoking, and obesity (OR=12.0).

Comparison of MetM patients *vs* healthy controls revealed

Table 4 Association of ≥20 years of smoking, obesity, *VDR*-BsmI genotype, and *VDR*-FokI genotype as single or combined with variables of melanoma.

Single or combined variable	Adjusted[1] OR (CI), *P* (All melanoma cases *vs*. healthy controls)	Adjusted[2] OR (CI), *P* (Melanomas *vs*. healthy controls)	Adjusted[1] OR (CI), *P* (MetM *vs*. healthy controls)	Adjusted[2] OR (CI), *P* (MetM *vs*. healthy controls)	Adjusted[1] OR (CI), *P* (NMetM *vs*. healthy controls)	Adjusted[2] OR (CI), *P* (NMetM *vs*. healthy controls)
≥20 years of ever-smoking	**2.19 (1.13–4.24), 0.020**	1.79 (0.85–3.74), 0.124	**3.16 (1.44–6.95), 0.004**	**2.46 (1.00–6.05), 0.050**	1.50 (0.69–3.28), 0.304	1.13 (0.47–2.73), 0.789
BMI >30 kg/m^2	**3.20 (1.12–9.14), 0.030**	**5.28 (1.73–16.1), 0.003**	**3.95 (1.22–12.8), 0.022**	**7.74 (2.02–29.6), 0.003**	2.51 (0.76–8.33), 0.132	**3.69 (1.04–13.1), 0.043**
BB	0.92 (0.51–1.66), 0.777	0.99 (0.52–1.91), 0.982	0.76 (0.34–1.68), 0.495	0.93 (0.39–2.25), 0.881	1.06 (0.54–2.08), 0.874	1.01 (0.48–2.12), 0.975
Bb	1.47 (0.87–2.49), 0.147	1.48 (0.83–2.63), 0.186	1.09 (0.56–2.12), 0.808	0.89 (0.41–1.92), 0.768	**1.88 (1.01–3.49), 0.045**	**2.15 (1.09–4.25), 0.028**
bb	0.66 (0.36–1.20), 0.171	0.61 (0.31–1.18), 0.140	1.15 (0.56–2.35), 0.705	1.21 (0.53–2.77), 0.644	**0.36 (0.16–0.81), 0.013**	**0.30 (0.12–0.75), 0.010**
BB+Bb (B allele)	1.52 (0.83–2.76), 0.171	1.65 (0.85–3.19), 0.140	0.87 (0.42–1.78), 0.705	0.82 (0.36–1.88), 0.644	**2.81 (1.24–6.36), 0.013**	**3.30 (1.34–8.15), 0.010**
Bb+bb (b allele)	1.09 (0.60–1.97), 0.777	1.01 (0.52–1.94), 0.982	1.32 (0.60–2.92), 0.495	1.07 (0.44–2.58), 0.881	0.95 (0.48–1.86), 0.874	0.99 (0.47–2.08), 0.975
bb plus ≥20 years of smoking	4.16 (0.86–20.2), 0.077^	3.24 (0.58–17.9), 0.179	**6.81 (1.27–36.6), 0.025**	**6.99 (1.08–45.2), 0.041**	1.68 (0.23–12.4), 0.612	0.98 (0.10–9.54), 0.987
Bb+bb plus ≥20 years of smoking	**2.08 (1.00–4.29), 0.049**	1.75 (0.78–3.92), 0.171	**3.29 (1.42–7.60), 0.005**	2.42 (0.93–6.28), 0.070^	1.19 (0.49–2.89), 0.702	0.94 (0.36–2.51), 0.910
bb plus BMI >30 kg/m^2	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
Bb+bb plus BMI >30 kg/m^2	**3.20 (1.00–10.3), 0.050**	**4.35 (1.28–14.8), 0.018**	**4.34 (1.22–15.5), 0.024**	**6.55 (1.60–26.9), 0.009**	2.26 (0.58–8.85), 0.242	2.81 (0.67–11.8), 0.158
Bb+bb plus ≥20 years of smoking, and plus BMI >30 kg/m^2	**8.61 (1.06–69.8), 0.044**	**12.0 (1.40–104), 0.024**	**17.4 (2.07–146), 0.008**	**22.7 (2.46–209), 0.006**	1.93 (0.12–32.1), 0.646	2.68 (0.14–49.8), 0.508
FF	0.80 (0.47–1.36), 0.413	0.90 (0.50–1.62), 0.732	0.59 (0.29–1.20), 0.145	0.60 (0.27–1.33), 0.206	0.97 (0.53–1.80), 0.935	1.12 (0.57–2.19), 0.749
Ff	1.30 (0.77–2.20), 0.317	1.21 (0.68–2.17), 0.516	1.70 (0.87–3.35), 0.122	1.59 (0.73–3.45), 0.243	1.11 (0.60–2.05), 0.728	1.03 (0.53–2.03), 0.923
ff	0.88 (0.39–1.98), 0.758	0.81 (0.33–1.96), 0.638	0.90 (0.32–2.54), 0.846	1.08 (0.34–3.41), 0.902	0.82 (0.31–2.14), 0.682	0.72 (0.25–2.03), 0.531
FF+Ff (F allele)	1.13 (0.51–2.55), 0.758	1.24 (0.51–3.01), 0.638	1.11 (0.39–3.12), 0.846	0.93 (0.29–2.95), 0.902	1.22 (0.47–3.21), 0.682	1.39 (0.49–3.96), 0.531
Ff+ff (f allele)	1.25 (0.73–2.12), 0.413	1.11 (0.62–1.99), 0.732	1.69 (0.83–3.43), 0.145	1.68 (0.75–3.74), 0.206	1.03 (0.55–1.89), 0.935	0.90 (0.46–1.75), 0.749
Ff plus ≥20 years of smoking	**2.77 (1.10–6.98), 0.031**	2.36 (0.84–6.58), 0.102	**4.02 (1.45–11.1), 0.007**	**3.69 (1.12–12.2), 0.032**	1.78 (0.59–5.39), 0.307	1.48 (0.42–5.23), 0.545
Ff+ff plus ≥20 years of smoking	**2.86 (1.24–6.60), 0.013**	2.30 (0.91–5.82), 0.080^	**4.21 (1.65–10.7), 0.003**	**3.92 (1.31–11.7), 0.014**	1.83 (0.68–4.93), 0.234	1.31 (0.42–4.11), 0.646
Ff plus BMI >30 kg/m^2	4.41 (0.92–21.1), 0.063^	**6.91 (1.35–35.4), 0.020**	**6.09 (1.11–33.4), 0.037**	**14.6 (2.16–98.3), 0.006**	3.61 (0.63–20.5), 0.148	4.39 (0.71–27.2), 0.112
Ff+ff plus BMI >30 kg/m^2	**6.09 (1.32–28.0), 0.020**	**8.89 (1.82–43.4), 0.007**	**8.50 (1.66–43.4), 0.010**	**17.8 (2.82–112), 0.002**	4.53 (0.85–24.3), 0.078^	5.63 (0.98–32.4), 0.053^

[1]Adjusted OR (CI) for gender, and age. [2]Adjusted OR for gender, age, phototype 1+2, total body nevi >50, and >10 lifelong sunburns. [a]OR uncountable because one of the compared group had zero subject. Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

that combination of Bb+bb and ≥20 years of smoking was significant (OR=3.29) after adjustment of type 1 confounders, but became a tendency after adjustment of type 2 confounders. By contrast, eight other variables were significant after both multivariate analyses of types 1 and 2 confounders. Specifically, by analysis of type 2 confounders, the following significant findings were observed: ≥20 years of smoking alone (OR=2.46) or combined with bb (OR=6.99), Ff (OR=3.69), and Ff+ff (OR=3.92) genotype; and obesity alone (OR=7.74) or combined with Bb+bb (OR=6.55), Ff (OR=14.6), and Ff+ff (OR=17.8) genotype. Notably, triple combination of Bb+bb, ≥20 years of smoking, and obesity resulted in type 2-adjusted OR=22.7 for MetM patients *vs* healthy controls.

Adjusted comparisons of type 2 confounders among NMetM patients *vs* healthy controls revealed risk effects of obesity (OR=3.69), Bb (OR=2.15), and BB+Bb (OR=3.30) genotypes.

Comparisons of *VDR*-BsmI and *VDR*-FokI genotypes, smoking, and obesity alone or their combinations (Table 5)

Table 5 illustrates comparison of MetM *vs* NMetM patients by adjusted analyses of types 1 to 5 confounders. Smoking duration of ≥20 years is a significant risk factor for MetM *vs*. NMetM patients (OR=2.26, 95% of CI=1.00–5.10, *P*=0.050) after adjustment for obesity (type 5 confounder). However, this risk factor became a tendency after extensive adjustments (types 1 and 2 confounders). Notably, bb genotype showed consistent risky adjusted OR=3 for MetM *vs*. NMetM after multivariate analysis of types 1 to 5 confounders. Consequently, carriage of B allele (i.e., BB+Bb) resulted in protective effects with respect to MetM. Significant threefold increased risk for MetM *vs*. NMetM cases was observed for the combination of Bb+bb and ≥20 years of smoking after adjustments for types 1, 2, and 5 confounders. Finally, triple combination of Bb+bb, ≥20 years of smoking, and obesity showed high types 1 and 2-adjusted ORs (OR=10.7 and OR=11.8, respectively), thus attesting for gene-behavioral effects among MetM patients.

Discussion

Our study was carried out under the context of precision medicine approach for disease treatment and prevention, which considers individual variability in genes, environment, and lifestyles[42].

VDR-BsmI polymorphism

We observed similar general distribution of *VDR*-BsmI genotypes (BB 25.0%, Bb 53.3%, bb 21.7%) among all

Table 5 Association of ≥20 years of smoking, obesity, *VDR*-BsmI genotype, and *VDR*-FokI genotype as single or combined variables with MetM (*n*=52) *vs*. NMetM (*n*=68), as evaluated by adjusted[1,2,3,4,5] OR (CI)

Single or combined variable	Adjusted[1] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[2] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[3] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[4] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[5] OR (CI), *P* (MetM *vs*. NMetM)
≥20 years of smoking	2.14 (0.92–4.94), 0.075^	2.14 (0.91–5.04), 0.081^	–[a]	–[a]	**2.26 (1.00–5.10), 0.050**
BMI >30 kg/m^2	1.60 (0.54–4.75), 0.398	1.82 (0.58–5.77), 0.306	–[a]	1.45 (0.48–4.37), 0.511	–[a]
BB	0.77 (0.32–1.85), 0.563	0.85 (0.35–2.05), 0.712	0.96 (0.34–2.77), 0.945	0.71 (0.30–1.69), 0.439	0.71 (0.30–1.66), 0.429
Bb	0.52 (0.24–1.12), 0.094^	0.47 (0.21–1.02), 0.058^	0.45 (0.18–1.12), 0.086^	0.59 (0.28–1.24), 0.166	0.59 (0.28–1.23), 0.159
bb	**3.28 (1.30–8.23), 0.012**	**3.42 (1.34–8.76), 0.010**	**3.06 (1.05–8.88), 0.040**	**3.16 (1.25–7.99), 0.015**	**3.17 (1.27–7.91), 0.013**
BB+Bb (B allele)	**0.30 (0.12–0.77), 0.012**	**0.29 (0.11–0.75), 0.010**	**0.33 (0.11–0.95), 0.040**	**0.32 (0.12–0.80), 0.015**	**0.31 (0.13–0.79), 0.013**
Bb+bb (b allele)	1.29 (0.54–3.09), 0.563	1.18 (0.49–2.87), 0.712	1.04 (0.36–2.98), 0.945	1.41 (0.59–3.35), 0.439	1.14 (0.60–3.32), 0.429
bb and ≥20 years of smoking	4.38 (0.85–22.7), 0.078^	4.38 (0.82–23.3), 0.083^	–[a]	–[a]	4.73 (0.92–24.2), 0.062^

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Continued

Single or combined variable	Adjusted[1] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[2] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[3] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[4] OR (CI), *P* (MetM *vs*. NMetM)	Adjusted[5] OR (CI), *P* (MetM *vs*. NMetM)
Bb+bb and ≥20 years of smoking	**2.79 (1.07–7.22), 0.035**	**3.02 (1.13–8.09), 0.027**	_[a]	_[a]	**2.92 (1.16–7.32), 0.023**
bb plus BMI >30 kg/m^2	3.22 (0.32–32.8), 0.323	3.37 (0.31–37.1), 0.320	_[a]	2.92 (0.28–30.4), 0.371	_[a]
Bb+bb plus BMI >30 kg/m^2	1.94 (0.57–6.55), 0.285	2.10 (0.59–7.42), 0.250	_[a]	1.66 (0.48–5.76), 0.426	_[a]
Bb+bb plus ≥20 years of smoking, and plus BMI >30 kg/m^2	**10.7 (1.26–90.7), 0.030**	**11.8 (1.33–105), 0.027**	_[a]	_[a]	_[a]
FF	0.65 (0.30–1.39), 0.269	0.68 (0.31–1.49), 0.341	0.65 (0.26–1.61), 0.353	0.64 (0.30–1.39), 0.261	0.64 (0.30–1.37), 0.249
Ff	1.43 (0.68–2.99), 0.342	1.46 (0.68–3.16), 0.332	1.01 (0.42–2.46), 0.974	1.48 (0.71–3.11), 0.296	1.48 (0.71–3.07), 0.291
ff	1.14 (0.35–3.72), 0.830	0.98 (0.28–3.36), 0.970	3.32 (0.73–15.1), 0.120	1.05 (0.32–3.43), 0.937	1.07 (0.33–3.43), 0.915
FF+Ff (F allele)	0.88 (0.27–2.87), 0.830	1.02 (0.30–3.53), 0.970	0.30 (0.07–1.37), 0.120	0.95 (0.29–3.11), 0.937	0.94 (0.29–3.02), 0.915
Ff+ff (f allele)	1.54 (0.72–3.29), 0.269	1.46 (0.67–3.18), 0.341	1.54 (0.62–3.82), 0.353	1.55 (0.72–3.34), 0.261	1.56 (0.73–3.33), 0.249
Ff plus ≥20 years of smoking	2.27 (0.79–6.48), 0.126	2.31 (0.79–6.72), 0.126	_[a]	_[a]	2.51 (0.91–6.97), 0.077^
Ff+ff plus ≥20 years of smoking	2.34 (0.89–6.13), 0.083^	2.34 (0.87–6.28), 0.093^	_[a]	_[a]	2.51 (0.99–6.39), 0.054^
Ff plus BMI >30 kg/m^2	1.46 (0.36–5.89), 0.593	1.59 (0.38–6.65), 0.521	_[a]	1.52 (0.37–6.16), 0.559	_[a]
Ff+ff plus BMI >30 kg/m^2	1.73 (0.51–5.92), 0.382	1.81 (0.51–6.44), 0.357	_[a]	1.67 (0.48–5.77), 0.421	_[a]

[1]Adjusted OR (CI) for gender, and age. [2]Adjusted OR for gender, age, phototype 1+2, total body nevi >50, and >10 lifelong sunburns.[3]Adjusted OR for trunk location, Breslow thickness, ulceration, mitosis >1, TILs absence, and epithelioid variant.[4]Adjusted OR for ≥20 years of smoking.[5]Adjusted OR for obesity (BMI >30 kg/m^2).[a]Non calculated to avoid over-correction as described in "Methods". Significant differences were indicated in bold, tendencies were evidenced with superscript ^.

melanoma patients and healthy controls (BB 25.8%, Bb 46.7%, bb 27.5%), showing agreement with other case-control investigations[16,20]. Notably, a threefold higher frequency of bb genotype was observed in MetM (32.7%) compared with NMetM cases (13.2%); this value ranged from significant crude OR=3.18 to adjusted ORs ranging from 3.06 to 3.42 after considering several confounders. We observed that B carriers (BB+Bb) were at reduced risk comparing MetM *vs*. NMetM cases. However, B carriers were at increased risk when comparing NMetM *vs*. healthy controls. Paradoxically, by comparison with healthy controls, carriage of bb genotype posed risk to MetM, but was protective for NMetM cases. In this study, distributions of genotypes in melanoma patients were similar with respect to Bb frequencies of those observed in central Italy by Santonocito et al.[15] in 101 melanoma patients (BB 9.9%, Bb 53.5%, bb 36.6%). The study indicated increased frequencies of Bb and bb genotypes in melanoma patients compared with healthy controls (BB 23.8%, Bb 50.5%, bb 25.7%) and demonstrated an association between *VDR*-BsmI bb genotype and increased Breslow's thickness[15], a parameter that is consistently associated with metastasis and poor prognosis[9]. A meta-analysis[17] showed that BsmI B allele is associated with reduced melanoma risk with OR=0.81 and 95% CI=0.72–0.92. A large-scale study of incidence of multiple primary melanoma revealed distribution of *VDR*-BsmI, with values of BB 18.9%, Bb 46.8%, and bb 34.2%, among patients with multiple primary melanomas and BB

15.3%, Bb 47.7%, and bb 37.0% among patients with single primary melanoma[19]. A recent meta-analysis[22] reported a 15% decrease in melanoma risk (pooled OR=0.85, 95% CI=0.76–0.94) for individuals with BB or Bb genotype compared with subjects featuring bb genotype.

In our study, bb genotype combined with ≥20 years of smoking yielded adjusted OR=7 for MetM patients *vs* healthy controls. Bb+bb (i.e., b allele) genotype combined with obesity showed adjusted ORs from 4 to 7 for MetM patients *vs* healthy controls.

Functional effect of *VDR*-BsmI polymorphism remains unclear[39,41,43]. This SNP is located in an intron sequence at the 3' end of *VDR* gene. Thus, *VDR*-BsmI polymorphism cannot directly change the protein sequence of the VDR receptor. Some studies suggested that this SNP can influence *VDR*-mRNA expression, thereby affecting its stability[43]. BsmI site may be in LD with other truly relevant SNPs in *VDR* or other genes[14,15,44].

VDR-FokI polymorphism

In our study, *VDR*-FokI genotypes (FF 39.2%, Ff 50.0%, and ff 10.8% in melanoma cases *vs*. FF 45.0%, Ff 41.7%, and ff 13.3% in healthy controls) were not associated with melanoma, and this result agrees with results of a recent meta-analysis[21]. However, we noted an increased risk for heterozygous Ff carriers when we compared MetM *vs*. NMetM cases. A Serbian study showed that compared with ff genotype, Ff and FF were associated with increased melanoma risk (OR=3.03, P=0.003; OR=9.28, P<0.001, respectively)[45]. In general, inconsistent findings were reported for association of *VDR*-FokI polymorphism with melanoma[19,46]. In one meta-analysis[20], FokI polymorphism was associated with an overall significantly increased risk of skin cancer (Ff *vs*. FF: OR=1.20, 95% CI=1.01–1.44; ff *vs*. FF: OR=1.41, 95% CI=1.08–1.84; Ff+ff *vs*. FF: OR=1.26, 95% CI=1.04–1.53). Another meta-analysis[22] claimed that f allele carriers showed an 18% (pooled OR=1.18, 95% CI=1.07–1.29) increased risk for melanoma compared with FF homozygotes. Notably, in our study, Ff+ff (f allele carriers), when combined with ≥20 years of smoking or with obesity, exhibited adjusted OR=4 and ORs from 8 to 18, respectively, for MetM patients *vs*. healthy controls. The f allele codes for a 427 amino acids long VDR protein, and it is considered less effective than the protein receptor coded by F allele (424 amino acids long)[40,41,43].

Smoking

Our study highlighted the crucial role of smoking duration in susceptibility to cutaneous melanoma and MetM. Past-smoking for ≥20 years resulted in fourfold risk factor for melanoma development with respect to healthy controls (OR=4.36) and fivefold risk factor for development of MetM with respect to NMetM (OR=5.31), whereas ≥20 years of smoking ever in life yielded OR=2.43 and OR=2.39, respectively. We also observed that ≥20 years of ever in life smoking combined with certain genetic traits, specifically, with bb, Bb+bb (b allele carriers), Ff, and Ff+ff (f allele carriers) are associated with significant crude ORs ranging from 4 to 9 for MetM cases *vs* healthy controls. Thus, smoke effects in melanoma can be modulated by VDR activity and by the pleiotropic vitamin D endocrine system[11-13,44]. Further studies are necessary to substantiate this significant and complex issue[10,11,44].

Despite the large number of studies[23,24,26-28], results on association of smoking with melanoma still present inconsistencies[23]. Some authors demonstrated risk effects of smoking in melanoma[10,23]. Using multivariate analysis (adjusted for age, sex, site of primary melanoma, and Breslow's thickness), a recent study by Newton-Bishop et al.[10] revealed that smoking duration at diagnosis (hazard ratio=1.11, 95% CI=1.03–1.20, P=0.009) is associated with risk of death from melanoma; and that lower vitamin D levels and smoking are associated with ulceration (a well-known poor prognostic factor) of primary melanomas and poor melanoma-specific survival[10]. We also noted positive association of ≥20 years of smoking with ulceration among melanoma patients. We similarly observed association of ≥20 years of smoking with TIL absence, a finding that predisposes in our and other studies to metastatic melanoma[8,9]. Conversely, other authors showed inverse relationship of smoking with melanoma[26-28]. Multiple potential confounders and biases can explain those protective associations of smoking[23]. Our present findings suggest that effects of smoking duration may be modulated by specific genetic traits.

In our study, past smokers among melanoma cases were twofold more frequent than among healthy controls. Our findings show association of ≥20 years of smoking with increased risk of melanoma, indicating the need for detailed assessment of lifelong smoke duration. We observed that among past smokers, MetM patients smoked approximately 8 years longer than NMetM patients (24.5±13.9 *vs*. 16.4±10.6 years). Notably, we demonstrated that ≥20 years of smoking serves as a two- to fivefold risk factor for MetM compared with NMetM patients. Among study participants, over 80% of past smokers with melanoma quit smoking before cancer diagnosis, with an average of 16 years before melanoma

development. This finding implies that exposure to smoke carcinogens requires long periods to induce melanoma onset and/or metastatic stage. Smoking effects long after smoking discontinuation provide intriguing evidence, which implies that some irreversible damages occur several years before first melanoma diagnosis. Long-lasting variations induced by smoking may include epigenetic changes in specific genes that can remain, for example, differentially methylated after smoking cessation (up to 22 years, as demonstrated by Ambatipudi et al.[47] and/or body accumulation of substances, such as heavy metals, including radionuclides Lead-210 and Polonium-210[48,49]. Explanation for such smoking phenomena require future detailed biological research and human studies. A recent study on melanoma cells observed a role for epigenetic mechanisms in VDR-miRNAs regulation[50].

In our study, ≥20 years of smoking combined with carriage of b allele (Bb+bb) showed adjusted OR=3 for MetM *vs*. NMetM patients after extensive multivariate analyses. This issue warrants further large-scale studies.

Coffee

We did not observe any significant findings in terms of coffee consumption. Thus, our data do not confirm protective effects of coffee consumption, as observed by other researchers[29].

Obesity

In our study, obesity presented an almost fourfold risk factor for melanoma susceptibility (OR=3.54), similar to previous population studies on malignant melanoma[26,33]. Obesity yielded an adjusted OR=5 for all melanoma patients *vs*. healthy controls and adjusted OR=8 for MetM patients *vs*. healthy controls. Obesity combined with Ff or Ff+ff exhibited high adjusted OR=15 and OR=18, respectively, for MetM patients *vs*. healthy controls. BMI is extensively evaluated in relation to several cancer types[51]. A large-cohort Italian study demonstrated that BMI≥25 kg/m^2 is associated with Breslow's thickness>1 mm among melanoma patients[9].

We are the first research group to assess the role of combination of obesity with specific *VDR* genetic traits in cutaneous melanoma. Interpretation of the association of obesity with melanoma may feature a biological rationale. Newton-Bishop et al.[10] hypothesized that inflammation associated with obesity can influence outcome of melanoma. Some evidence also showed the genetic link between obesity and pigmentation or hair color[52].

Triple combination of *VDR* genetic traits, smoking, and obesity

In our study, the highest ORs were observed after combination of a *VDR* genetic trait (b allele carriers) and two lifestyle parameters, i.e., Bb+bb plus ≥20 years of smoking and plus obesity by comparing all melanoma patients *vs*. healthy controls (OR=9.65), MetM patients *vs*. healthy controls (OR=21.6), and MetM *vs*. NMetM patients (OR=12.2). All data remained significant according to multivariate analyses.

However, we failed to calculate ORs for analogous triple combination comprising f allele carriers, because six study subjects with Ff+ff plus ≥20 years of smoking and plus obesity were all MetM patients. Further large-scale studies are necessary for such assessments.

Roles of vitamin D in melanoma require further studies. Melanoma cell culture and xenograft experiments in mice highlighted that vitamin D poses tumoral and metastasis suppression effects[53-55]. Virtually all actions of vitamin D occur through VDR activation. Thus, any modification of VDR activities induced by *VDR* polymorphisms can affect vitamin D functions[13]. Deletion of VDR results in increased susceptibility to tumor formation and reduces ability of keratinocytes to clear UVB-induced DNA mutations[13,56]. VDR can bind to thousands of VDREs on human genome and up- or down-regulate hundreds of genes. Of interest, recent evidence showed a crosstalk between VDR and immune factors[57]. VDR cistrome analyses suggested that altered expression of VDR in colon cancer changes actions of VDR, thus affecting patient outcome[58]. A recent study showed that *VDR* genetic traits can modulate VDR protein expression in excised human melanoma tissues, which might have implications for effects of vitamin D activity on melanoma cells[59].

Thus, future research should focus on complex gene interactions and biological pathways related to vitamin D, VDR, smoking, excessive fat, and environmental factors with melanoma. Improved comprehension of biomolecular pathways will support further progress in melanoma management[60].

Study limitations and strengths

Limitations of our study include limited number of melanomas and high CIs for some categorical variables. Nonetheless, several ORs were statistically significant. Analysis by data stratification for combined variables in some cases resulted in comparison of groups with less than 10

subjects. Thus, future large-scale studies are necessary to better assess the role of such combined variables. We focused on white residents in northern Italy. Thus, our results cannot be generalized to populations with different genetic backgrounds. By contrast, a critical strength of our study is highly defined ethnic background of subjects. This variable bears significance in genetic studies. Variability in racial distribution and genetic melanoma susceptibility among (and across) different countries suggests that melanoma studies should be performed in restricted and well-characterized ethnic groups[7]. Another strength of our study is the detailed reported information, including combinations of genetic and lifestyle factors.

Conclusions

Treatment-resistant metastatic cancer is the most significant contributor to cancer mortality worldwide. Thus, better understanding of factors contributing to development of metastatic cancer may increase likelihood of future improvements in patient management. Our data highlighted that in terms of *VDR* gene alteration by SNPs, vitamin D homeostasis plays roles in cutaneous melanoma and MetM, and these functions are further enhanced by individual smoking habits and BMI. Thus, our findings support a gene-environment contribution to development of malignant melanoma, suggesting the value of genetic screening, smoking cessation[10], and excessive fat prevention[51].

We first suggest gene-environment effects, including smoking duration and obesity, and *VDR* genetic polymorphisms with cutaneous malignant melanoma in general and specifically with MetM. Current data may contribute to development of a personalized/precision management for melanoma patients. Such management may include screening of *VDR* polymorphisms and detailed assessment of smoking habits and BMI. Further investigations are necessary to substantiate and extend our findings to examine different ethnic groups and to identify biological pathways related to vitamin D, smoking, and excessive fat, which influence skin cancers.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

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