Vitamin D Receptor Polymorphisms Are Associated with Altered Prognosis in Patients with Malignant Melanoma[1]

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ABSTRACT

Calcitriol [1,25(OH)$_2$D$_3$], the hormonal derivative of vitamin D$_3$, is an antiproliferative and prodifferentiation factor for several cell types, including cultured melanocytes and malignant melanoma (MM) cells. Several polymorphisms of the vitamin D receptor (VDR) gene have been described including a *Fok*I RFLP in exon 2, *Bsm*I, and *Apa*I polymorphisms in intron 8 and an adjacent *Taq*I RFLP in exon 9. Alterations in vitamin D/1,25(OH)$_2$D$_3$ levels and polymorphisms of the VDR have been shown to be associated with several systemic malignancies. We hypothesize that polymorphism in this gene may be associated with altered susceptibility and outcome in patients with MM. A hospital-based case-control study, using 316 MM cases and 108 controls, was used to assess associations with MM susceptibility. Breslow thickness, the most important single prognostic factor in MM, was used as the outcome measure. Polymorphisms at the *Fok*I and *Taq*I restriction sites were determined using PCR-based methods. Polymorphism at the *Fok*I, but not *Taq*I, RFLP was associated with an altered risk of MM ($P = 0.014$). More importantly, variant alleles were associated with increased Breslow thickness. Thus, homozygosity for variant alleles at both RFLP (*ttff* genotype combination) was significantly associated with thicker tumors. (≥3.5 mm; $P = 0.001$; odds ratio = 31.5). Thus, polymorphisms of the *VDR* gene, which would be expected to result in impaired function, are associated with susceptibility and prognosis in MM. These data suggest that 1,25(OH)$_2$D$_3$, the ligand of the VDR, may have a protective influence in MM, as has been proposed for other malignancies.

INTRODUCTION

MM[4] is the most serious cutaneous malignancy, and the prognosis of some tumors is very poor (1, 2). It is predominantly a disease of white-skinned people, and exposure to UV light is thought to be critical, although the relationship between risk and exposure is unclear (2). Other important risk factors for the occurrence of MM include presence of excessive numbers of banal nevi, multiple atypical nevi, fair skin, red hair, and blue or green eyes.

Breslow thickness at presentation remains the most important single prognostic factor for patients with cutaneous MM (3). In general, patients with thin tumors have a much longer survival than those with thick lesions; the 5-year survival rate for lesions <1.5-mm thick is 93%, compared with 67% for 1.5 mm-3.49 mm and 37% for ≥3.5 mm (4). Risk factors for thicker tumors, and hence poorer prognosis, include age at initial presentation and tumor site.

Relatively little is known of the genetic factors that mediate susceptibility to, and outcome of, sporadic MM. Several putatively important genes, including the susceptibility genes *melanocyte stimulating hormone receptor* (5, 6), *glutathione S-transferase GSTM1* (7), and *cytochrome P450 CYP2D6* (8, 9) as well as the cancer candidate genes, *p16^{INK4a}* and *p15^{INK4b}* (10), have been studied, although thus far only the *CYP2D6 PM* genotype has been associated with increased risk in independent studies.

We propose that the *VDR* gene may influence susceptibility and outcome in MM. This view is supported by data showing that 1,25(OH)$_2$D$_3$ (the hormonal derivative of vitamin D$_3$ and the ligand of the VDR) has antiproliferative and prodifferentiation effects in VDR-expressing cell types (11–14). Furthermore, associations have been identified between 1,25(OH)$_2$D$_3$ and susceptibility to, and outcome of, systemic malignancies such as breast, prostate, and colon. These include association with both serum vitamin D/1,25(OH)$_2$D$_3$ levels as well as with polymorphisms in the *VDR* gene (15–19).

Similar supportive data exist for MM. Thus, melanocytes and MM cells express the VDR, and 1,25(OH)$_2$D$_3$ has an antiproliferative effect *in vitro* (20, 21). For example, stimulation of tyrosinase activity, a specific prodifferentiation stimulus, has been reported in melanocytes exposed to 1,25(OH)$_2$D$_3$ (21). *In vivo*, there is currently little evidence of involvement of vitamin D$_3$, although low serum levels of 1,25(OH)$_2$D$_3$ have been reported in

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[4] The abbreviations used are: MM, malignant melanoma; VDR, vitamin D receptor; OR, odds ratio; 95% CI, 95% confidence interval; calcitriol, 1,25(OH)$_2$D$_3$; CDK, cyclin-dependent kinase.

patients with MM (22). The role of sun exposure in MM is unclear. Current literature remains controversial, with most clinicians advocating a causative association between UV exposure and risk, whereas other studies support the view of a possible protective effect of vitamin D (generated at least in part by UV). For example, use of sunscreens is associated with increased MM risk, an all-year tan appears protective, and outdoor occupation appears to demonstrate no association with susceptibility to MM.

Five polymorphic sites have been identified in the VDR. These comprise RFLP in exon 2 (*Fok*I restriction site), the last intron (*Bsm*I and *Apa*I restriction sites), and an adjacent area of exon 9 (*Taq*I restriction site) as well as a poly(A) microsatellite length polymorphism in the 3′ untranslated region. The *Fok*I polymorphism results in an altered translation start site and has been shown to be functionally relevant (23). The other four sites demonstrate linkage disequilibrium, and there is evidence to suggest functional consequences of these polymorphisms (24).

Because these data support the view that polymorphism in the VDR gene may be an important determinant of susceptibility and outcome in patients with MM, the aim of the present study was to investigate the relationship between the VDR polymorphisms and susceptibility to and prognosis (as estimated by Breslow thickness) of MM. Because there is no evidence of linkage disequilibrium between the *Fok*I RFLP and the cluster of polymorphisms at the 3′ end of the gene and there is evidence to suggest functional consequences of each of these polymorphic regions, we have concentrated on the *Fok*I polymorphism and a representative example of the 3′ cluster (*Taq*I RFLP).

PATIENTS AND METHODS

Patients. All MM cases ($n = 316$) were of Northern European Caucasian extraction, originally presented between January 1994 and December 1997 and attended the Dermatology Departments at the Leicester Royal Infirmary, North Staffordshire Hospital or Royal Cornwall Hospitals, between 1996 and 1997. All tumors were histologically diagnosed as *in situ* or invasive MM. Lentigo maligna and lentigo maligna melanoma were not included, in view of the biological singularity of lentigo maligna. Patients with acral tumors or those with MM and other malignant pathologies (cutaneous or internal) were also excluded. We attempted to recruit all eligible patients, although some were randomly lost in busy clinics. None of the subjects approached refused to participate. This cohort comprises ~80% of all eligible patients and represents a typical sample of MM patients presenting to dermatologists in the participating centers. The controls ($n = 108$) comprised randomly recruited, hospital-based Northern European Caucasians attending these Dermatology departments with basal cell papillomas and without clinical or histological evidence of malignancy. Subjects with a history of inflammatory pathology were also excluded. The study was performed with local Ethical Committee approval, and informed consent was obtained from all of the individuals recruited.

Cases and controls were interviewed by a dermatologist (J. E. O., J. T. L., A. G. S., or P. W. B.). The following demographic and clinical data were recorded: patient age at presentation, gender, skin type in terms of propensity to sun burning and tanning using the Fitzpatrick classification (25), eye and hair color at age 21 years, tumor site, and Breslow thickness. Breslow thickness (defined as the vertical thickness of the tumor from the granular layer of the epidermis to the deepest part of the melanoma) was determined by specialist pathologists. On the basis of Breslow thickness, patients were divided into five categories: *in situ*, <0.75 mm, 0.75–1.49 mm, 1.5–3.49 mm, and ≥3.5 mm. Table 1 shows the distribution of these clinical parameters in the total case group. As also indicated in Table 1, complete clinical data could not be obtained from all patients because of insufficient time in busy clinics (74–95% for *Taq*I and 72–92% for *Fok*I genotyped cases).

Determination of VDR Genotype. All genotyping assays were performed by workers who were unaware of the clinical status of individual subjects. PCR assays to identify VDR genotypes included one DNA sample (selected at random) of known genotype for each batch of eight samples of unknown genotype, at least one homozygous variant DNA (*tt* or *ff*) as a control for restriction enzyme digestion, one negative control (no DNA), and molecular weight markers. Approximately 15% of all patient DNA samples were re-assayed on at least one occasion, and the genotype assignment was confirmed. All assignments were validated by an independent, blinded observer examining the agarose gels. DNA was extracted from peripheral blood (5 ml; collected into EDTA) using standard phenol-chloroform methods. PCR- RFLP based assays were used to identify alleles containing the exon 2 (*Fok*I) and exon 9 (*Taq*I) variants. Primers were selected based on the methods of Gross *et al.* (Ref. 26; *Fok*I) and Spector *et al.* (Ref. 27; *Taq*I) with modifications. The exon 2 wild-type (*F*) and variant (*f*) alleles were identified using primers 5′-AGCTGGCCCTGGCACTGACTCT-GCTCT-3′ and 5′-ATGGAAACACCTTGCTTCTTCTCCCT-C-3′ to amplify a 265-bp product. Amplification of template DNA was performed in an incubation mixture (total volume, 50 μl) comprising 20 pmol each of primer, 200 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, and 1 unit of *Taq* polymerase in buffer containing 10 mM Tris-HCl^- (pH 9.0), 50 mM KCl, and 0.1% (w/v) Triton X-100. The PCR conditions were: initial denaturation (94°C for 3 min), followed by 30 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 30 s), followed by a final extension at 72°C for 5 min. PCR products were then digested with *Fok*I (37°C for 20 h), and the products were examined after electrophoresis in 2% agarose gels. The *F* allele was refractory to digestion, whereas *f* was identified by fragments of 196 and 69 bp. The *Taq*I wild-type (*T*) and variant (*t*) alleles were identified using the forward primer from Spector *et al.* (27), 5′-CAGAGCATGGACAGGGAGCAAG-3′, and a novel reverse primer, 5′-CGGCAGCGGATGTACGTCTGCAG-3′, to amplify a 345-bp PCR product. The PCR conditions were as for the *Fok*I RFLP. PCR products were then digested with *Taq*I (65°C for 20 h), and the products were examined after electrophoresis in 2% agarose gels. The *T* allele was refractory to digestion, whereas *t* was identified by fragments of 260 and 85 bp. We attempted to obtain genotype data from all samples, but in some earlier cases, DNA was exhausted or refractory to amplification.

Statistical Analysis. Statistical analysis was undertaken using the Stata software package (version 5.0; Stata Corp., College Station, TX). χ^2 tests were used to test for homogeneity between and within cases and controls (28). Because some frequencies were small, the StatXact-Turbo statistical package (version 3; Cytel Software Corp., Cambridge, MA) was used to obtain exact significance levels (*P*s). Logistic regression analysis was used to examine differences between cases and controls while simultaneously

Table 1 Frequency of *Taq*I and *Fok*I polymorphisms in controls and MM cases[a]

		*Taq*I				*Fok*I		
	n	*TT*	*Tt*	*tt*	*n*	*FF*	*Ff*	*ff*
Controls	93	39 (41.9%)	41 (44.1%)	13 (14.0%)	108	52 (48.1%)	44 (40.7%)	12 (11.1%)
MM cases[b]	261	94 (36.0%)	127 (48.7%)	40 (15.3%)	293	105 (35.8%)	142 (48.5%)	46 (15.7%)
MM cases								
Site								
Head/Neck	21	4 (19.1%)	14 (66.7%)	3 (14.3%)	22	13 (59.1%)	9 (40.9%)	0 (0.0%)
Trunk	85	31 (36.5%)	40 (47.1%)	14 (16.5%)	98	29 (29.6%)	50 (51.0%)	19 (19.4%)
U Limbs	35	14 (40.0%)	14 (40.0%)	7 (20.0%)	41	18 (43.9%)	17 (41.5%)	6 (14.6%)
L Limbs	99	33 (33.3%)	51 (51.5%)	15 (15.2%)	108	36 (33.3%)	57 (52.8%)	15 (13.9%)
	240				269			
Skin type								
1	58	18 (31.0%)	32 (55.2%)	8 (13.8%)	64	20 (31.3%)	36 (56.3%)	8 (12.5%)
2	146	53 (36.3%)	66 (45.2%)	27 (18.5%)	152	56 (36.8%)	71 (46.7%)	25 (16.5%)
3	35	14 (40.0%)	17 (48.6%)	4 (11.4%)	41	15 (36.6%)	20 (48.8%)	6 (14.6%)
4	5	2 (40.0%)	2 (40.0%)	1 (20.0%)	9	2 (22.2%)	5 (55.6%)	2 (22.2%)
	244				266			
Eye color								
Brown	54	19 (35.2%)	25 (46.3%)	10 (18.5%)	58	20 (34.5%)	28 (48.3%)	10 (17.2%)
Blue	141	50 (35.5%)	70 (49.7%)	21 (14.9%)	154	58 (37.7%)	76 (49.4%)	20 (13.0%)
Green	38	19 (39.5%)	16 (42.1%)	7 (18.4%)	43	11 (25.6%)	24 (55.8%)	8 (18.6%)
Hazel	14	3 (21.4%)	10 (71.4%)	1 (7.1%)	15	5 (33.3%)	6 (40.0%)	4 (26.7%)
	247				270			
Hair color								
Red	22	7 (31.8%)	15 (68.2%)	0 (0.0%)	23	9 (39.1%)	14 (60.9%)	0 (0.0%)
Blonde	34	12 (35.3%)	16 (47.1%)	6 (17.7%)	40	14 (35.0%)	22 (55.0%)	4 (10.0%)
Brown	136	52 (38.2%)	62 (45.6%)	22 (16.2%)	145	49 (33.8%)	64 (44.1%)	32 (22.1%)
Black	1	0 (0.0%)	1 (100.0%)	0 (0.0%)	3	1 (33.3%)	2 (66.7%)	0 (0.0%)
	193				211			
Breslow[c]								
In situ	40	13 (32.5%)	22 (55.0%)	5 (12.5%)	46	14 (30.4%)	23 (50.0%)	9 (19.6%)
0.1–0.74 mm	72	28 (38.9%)	33 (45.8%)	11 (15.3%)	75	27 (36.0%)	35 (46.7%)	13 (17.3%)
0.75–1.4 mm	47	18 (38.3%)	24 (51.1%)	5 (10.6%)	62	24 (38.7%)	29 (46.8%)	9 (14.5%)
1.5–3.4 mm	35	9 (25.7%)	18 (51.4%)	8 (22.9%)	38	13 (34.2%)	19 (50.0%)	6 (15.8%)
≥3.5 mm	12	4 (33.3%)	4 (33.3%)	4 (33.3%)	14	2 (14.3%)	8 (57.1%)	4 (28.6%)
	206				235			

[a] Analysis was performed using logistic regression.

[b] Proportion of subjects with the *FF* genotype in cases *versus* controls; $P = 0.026$; OR, 0.60; 95% CI, 0.38–0.94 (uncorrected); and $P = 0.029$; OR, 0.59; 95% CI, 0.37–0.95 (corrected for age and gender).

[c] Proportion of patients with the *tt* genotype in MM cases with a Breslow thickness of ≥1.5 mm compared with <1.5 mm; $P = 0.047$; OR, 2.25; 95% CI, 1.01–5.02 (uncorrected); and $P = 0.131$; OR, 1.92; 95% CI, 0.82–4.48 (corrected for age and gender).

correcting for imbalances in age and gender. Logistic regression was also used to examine differences in genotype frequencies between cases stratified by Breslow thickness, while correcting for age at presentation and gender. Significant associations of combined genotypes (*e.g.*, *ttff*) were only accepted if they remained significant in the presence of the main effects (*i.e.*, a model including *ttff*, *tt*, and *ff*). If the significance of the combined genotype disappeared, this would suggest that the factors were acting independently, and the significance of the combined effect was driven by the strength of either (or both) of the component factors. Associations of Breslow thickness were confirmed using linear regression after transformation of thickness values to normality and correction for age at presentation and gender. Because some tumors were *in situ* (0 mm thick), transformation was performed using the formula: ln (Breslow thickness + 1).

RESULTS

Three hundred and sixteen patients with MM (mean age ± SD, 53.3 ± 16.7 years; 67% female) and 108 controls (mean age ± SD, 55.7 ± 19.7 years; 50% female) were recruited.

Case-Control Analysis. Table 2A shows the allele frequencies of *Taq*I and *Fok*I alleles in controls and MM cases. All allele frequencies conformed to Hardy-Weinberg equilibrium. The *F* allele was significantly less common in MM cases than controls ($P = 0.029$; OR, 0.69; 95% CI, 0.50–0.96). Table 2B shows the relationship between *Taq*I and *Fok*I genotypes in cases and controls. No significant correlations between genotypes at the two sites were identified in either controls ($P = 0.365$) or cases ($P = 0.847$), suggesting that the two polymorphisms did not demonstrate linkage disequilibrium. Table 1 shows frequencies of *Fok*I and *Taq*I genotypes in controls compared with MM cases. There was a decreased proportion of individuals with the *Fok*I *FF* genotype in cases *versus* controls. Thus, for *FF versus* other *Fok*I genotypes, the uncorrected OR for MM was 0.60 ($P = 0.026$). The findings remained significant after correction for age and gender using multivariate logistic regression (OR, 0.59; $P = 0.029$). The estimated risk reduction attributable to the *FF* genotype was 23.7% (95% CI, 1.2–51.3%).

Table 2 Frequency of *Taq*I and *Fok*I alleles and concordance between genotypes in controls and MM cases

A. Allele frequencies

	*Taq*I			*Fok*I		
	n	T	t	*n*	F	f
Controls	186	119 (64.0%)	67 (36.0%)	216	148 (68.5%)	68 (31.5%)
MM cases[a]	522	315 (60.3%)	207 (39.7%)	586	352 (60.1%)	234 (39.9%)

B. Concordance

	Controls			MM Cases		
	*Taq*I			*Taq*I		
*Fok*I	*TT*	*Tt*	*tt*	*TT*	*Tt*	*tt*
FF	17 (47.2%)	12 (33.3%)	7 (19.4%)	30 (32.3%)	49 (52.7%)	14 (15.0%)
Ff	17 (43.6%)	17 (43.6%)	5 (12.8%)	44 (36.1%)	57 (46.7%)	21 (17.2%)
ff	3 (33.3%)	6 (66.7%)	0 (0.0%)	16 (40.0%)	19 (47.5%)	5 (12.5%)

[a] Proportion of subjects with the *F* allele in cases *versus* controls; $P = 0.029$; OR, 0.69; 95% CI, 0.50–0.96.

Association of VDR Genotype with Patient Characteristics. Table 1 shows the frequency of VDR genotypes in the MM cases stratified by patient characteristics. There was no significant association between genotype frequencies and tumor site, skin type, or eye color. However, the *ff* and *tt* genotypes were significantly less common in MM patients with red hair than in patients with other hair colors ($P = 0.021$, $\chi^2_1 = 5.31$ and $P = 0.040$, $\chi^2_1 = 4.21$, respectively).

Association for VDR Genotypes with Breslow Thickness. Patients were categorized by Breslow thickness (Table 1). In tumors ≥1.5-mm thick, for *Taq*I, there was an increased proportion of the *tt* genotype ($P = 0.047$), but there was no obvious effect for *Fok*I ($P = 0.701$). Homozygosity for variant alleles at either *Fok*I and *Taq*I loci (*tt* or *ff* genotypes) was associated with an increased proportion of tumors ≥3.5 mm thick, although this did not achieve statistical significance (*tt*: $P = 0.105$; OR, 2.84; and *ff*: $P = 0.266$; OR, 1.99, uncorrected).

The effects of combinations of the *Taq*I and *Fok*I polymorphisms are shown in Table 3. There was an association of *ttff* combined genotype with thicker tumors, using either ≥1.5 mm ($P = 0.065$) or ≥3.5 mm ($P < 0.001$) as the cutoff. These results retained similar significance, particularly for tumors ≥3.5-mm thick, after correction for potential confounding factors (age, gender, and tumor site). Thus, the mean Breslow thickness in patients with the *ttff* genotype combination was 2.9 mm compared with 1.1 mm in patients with other genotype combinations. This association was further confirmed using linear regression analysis, which showed that the *ttff* genotype was correlated with Breslow thickness ($P = 0.002$, transformed to normality and corrected for age and gender). Significant associations were also identified between Breslow thickness and combinations of genotypes including the genotypes *Ttff* and *ttFf*, although these were less effective at predicting Breslow thickness.

DISCUSSION

We have postulated that polymorphism in the *VDR* gene is important in MM. This hypothesis is supported by data showing that $1,25(OH)_2D_3$ inhibits cell proliferation (12, 13) and stimulates differentiation (11, 14) and apoptosis (29) in several cell types expressing the VDR. There is evidence that $1,25(OH)_2D_3$ has an anticancer effect in several systemic cancers such as breast (30), prostate (31), colon (32), leukemia (33), and kidney (34). Furthermore, *in vitro* studies have demonstrated that $1,25(OH)_2D_3$ inhibits growth of cultured malignant cells (11–14, 34) and inhibits experimental carcinogenesis (35, 36). *In vivo*, decreased mean serum levels of $1,25(OH)_2D_3$ or its precursors have been reported in carcinoma of the breast (15), prostate (17), and colon (19). More recently, polymorphisms of the VDR have been reported associated with cancer of the breast [*Fok*I and poly(A) site RFLP; Ref. 16) and prostate [*Bsm*I and poly(A) site RFLP; Refs. 18 and 37).

Data for MM are similar, although more limited. Normal (38) and malignant melanocytes (20) express the VDR, and $1,25(OH)_2D_3$ has been shown to inhibit normal (21) and malignant melanocyte (20) growth *in vitro*. In a study of $1,25(OH)_2D_3$ serum levels in MM patients, lower levels were found compared with controls, although this did not achieve statistical significance (22).

In our study, homozygosity for the wild-type (*F*) allele at the *Fok*I restriction sites was associated with a reduced risk of MM, with a risk reduction attributable to the *FF* genotype estimated at 23.7%. Furthermore, the proportion of *F* alleles was significantly lower in the case group compared with controls. The number of controls, however, was relatively small, and larger cohorts would be required to reduce the risk of both type 1 and type 2 errors. In this initial study, we have used hospital-based controls. Selection of control subjects is always difficult, and although the use of "normal" volunteers or blood donors would reduce the risk of potential bias because of occult associations with other disease processes, since they are generally not examined by a clinician, the possibility of undetected malignant or inflammatory pathologies cannot be excluded. By use of hospital-based controls, it was possible to focus only on controls who were clinically free of other malignant or inflammatory pathologies. Furthermore, the control genotype frequencies were similar to those described in other studies (18, 26, 27, 39), supporting the view that our control group is representative of the normal population.

The *Fok*I RFLP has been reported previously to be associated with breast cancer (16), where the *FF* genotype was

Table 3 Interactions between VDR genotypes and association with Breslow thickness

Significant associations of combined genotypes (*e.g.*, ttff) were only accepted if they remained significant in the presence of the main effects (*i.e.*, a model including ttff, tt, and ff). The reference category is all other genotype combinations (*e.g.*, all other patients except those with ttff).

A. <1.5 *vs.* 1.5 mm

Genotype combination	<1.5 mm	≥1.5 mm	*P*	OR	95% CI
ttff[a]	2/158 (1.3%)	3/45 (6.7%)	0.065	5.6	0.9–34.4
ttff[b]			0.023	9.2	1.4–61.8
ttff[c]			0.062	7.2	0.9–57.2
ttff or ttFf[a]	10/158 (6.3%)	9/45 (20.0%)	0.008	3.7	1.4–9.8
ttff or ttFf[a]			0.009	3.9	1.4–11.0
ttff or ttFf[c]			0.007	4.3	1.5–12.5
ttff or Ttff[a]	17/158 (10.8%)	6/45 (13.3%)	0.631	1.3	0.5–3.5
ttff or Ttff[b]			0.292	1.8	0.6–5.1
ttff or Ttff[c]			0.336	1.7	0.6–5.4

B. <3.5 mm *vs.* 2.3 mm

Genotype combination	<3.5 mm	≥3.5 mm	*P*	OR	95% CI
ttff[a]	2/191 (1.1%)	3/12 (25.0%)	<0.001	31.5	4.7–212.7
ttff[b]			<0.001	93.2	9.4–926.6
ttff[c]			<0.001	108.5	8.2–1438.8
ttff or ttFf[a]	16/191 (8.4%)	3/12 (25.0%)	0.071	3.6	0.9–14.8
ttff or ttFf[b]			0.075	3.8	0.9–16.4
ttff or ttFf[c]			0.090	4.8	0.8–29.5
ttff or Ttff[a]	19/191 (9.9%)	4/12 (33.3%)	0.022	4.5	1.2–16.4
ttff or Ttff[b]			0.005	7.8	1.8–32.9
ttff or Ttff[c]			0.006	12.3	2.1–73.1

[a] Uncorrected data.
[b] Corrected for age at presentation and gender.
[c] Corrected for age at presentation, gender, and head/neck tumor site.

associated with a decreased risk of ~50% in certain racial groups. The poly(A) polymorphism (classified into long, *L*, or short, *S*) has been associated with altered risk of breast (16) and prostate (18) cancer. In breast cancer, *LL* and *LS* alleles were also associated with a ~50% reduction in risk (16). However, in prostate cancer, the presence of *L*, whether in the heterozygous (*LL*) or homozygous (*LS*) state, was associated with a 4–5-fold increased risk of prostate cancer (18, 37). Because the *Taq*I restriction site is in strong linkage disequilibrium with the poly(A) polymorphism (*T* demonstrates linkage disequilibrium with *L*; Ref. 39), the findings in breast cancer are comparable with our findings in MM, although our data on the *Taq*I RFLP did not achieve statistical significance.

Our data also identified an association between VDR genotypes and red hair in patients with MM. There was insufficient hair color data on our control subjects to examine whether this was a general phenomenon. Although the mechanism for this association is not known, other studies have identified links between polymorphism at other loci and hair color in MM (6, 8). These data suggest that the molecular route by which patients with red hair develop MM may differ from patients with other hair colors, supporting the view that these patients represent a high risk subgroup. However, these data require confirmation in independent studies, including in control individuals.

More significantly, we have identified significant associations between VDR genotypes and outcome in patients with MM. Thus, our data suggest that VDR polymorphism is a better determinant of outcome in MM than of its initiation. Melanoma depth is well recognized as an important prognostic indicator with respect to risk of metastatic disease and survival (40). In general, for both restriction sites, the proportion of thick MMs (with either ≥1.5 or ≥3.5 mm cutoff) increased with increasing number of variant alleles. The effect of VDR genotypes on Breslow thickness was markedly increased when the two polymorphic sites were considered together. Thus, the combined *ttff* genotype was associated with tumors ≥1.5-mm thick but particularly those ≥3.5-mm thick ($P < 0.001$; Table 3). We also corrected the data for the potential confounding effects of gender, tumor site, and age at presentation because thicker tumors are associated with male gender, head/neck tumor site, and older age. The association of the *ttff* genotype remained significant, suggesting that the effect on Breslow thickness is independent of these factors. Similar results were obtained with other genotype combinations, although the magnitudes of the effects were smaller, suggesting that the heterozygote genotypes were of intermediate importance in determining Breslow thickness. Similarly, in carcinoma of the prostate, poly(A) microsatellite variants are reported to be associated with more advanced disease (37). In addition, low serum levels of $1,25(OH)_2D_3$ have been implicated in metastatic rather than *in situ* disease in prostatic cancer, suggesting an impact on tumor progression rather than development (17).

The polymorphism at the *Fok*I restriction site (T-C transition) produces an ATG start codon resulting in translation initiation 10 bp upstream and therefore the production of a lengthened protein of 427 amino acids (26, 41). The *F* allele (restriction site absent, ACG), which results in a shorter protein, has been shown to be more effective at activating the transcrip-

tion of a VDR reporter construct (23), thereby indicating that the polymorphism is functionally significant. The cluster of polymorphisms at the 3′ end of VDR, which includes *Taq*I, are in mutual tight linkage disequilibrium and a representative, *Bsm*, is known to be in linkage disequilibrium with the poly(A) microsatellite (39). It has been suggested that the length of the poly(A) repeat affects mRNA stability or is tightly linked to a further functionally significant site (24). The net effect of the *ff* and the *tt* polymorphisms can be envisaged as a reduction in the cellular effect of $1,25(OH)_2D_3$ and therefore a growth advantage of the melanocytes. This conclusion is supported by the increased effect of combined homozygosity, which would be expected to have a more profound effect on the VDR protein.

These data support the hypothesis that the *VDR* genotype has a significant role in determining tumor occurrence and behavior in MM and indicate a role for vitamin D in melanoma cell cycle control and differentiation *in vivo*. There is evidence of a blocking effect of $1,25(OH)_2D_3$ at the transition from G_1 to S phase of the cell cycle via several mechanisms, such as stimulation of the CDK inhibitory proteins, P21 (42), which contains a VDR response element (43), and P27 (44), and inhibition of cyclin D_1 (45). It is of interest that the other reported genetic changes associated with melanoma also have an impact at the G_1 to S-phase check point. These include mutations of the CDK inhibitor genes, $p16^{INK4a}$ (46–48) and $p15^{INK4b}$ (10), and mutations in the *CDK4* gene (49, 50). This point in the cell cycle may therefore be of pivotal importance in the development and/or progression of MM.

It has been argued above that the effect of the VDR polymorphisms, reported here, is a functional, cellular deficiency of $1,25(OH)_2D_3$. Decreased serum levels of $1,25(OH)_2D_3$ have been reported in certain cancers (15, 17, 19), including MM (22). Furthermore, some studies suggest that vitamin D deficiency, resulting from decreased cutaneous production from solar irradiation, may be contributory to the development of carcinoma of the breast (51), prostate (52), and colon (53). The role of sun exposure in MM is, however, more complex. On the one hand, it is firmly held by the majority of clinicians that solar radiation is causative in MM, and on the other, there is the potential deleterious effect of vitamin D deficiency, a cause of which is lack of sun exposure. Inhibition of MM cell growth *in vitro* by $1,25(OH)_2D_3$ (20), the effect of VDR polymorphisms described in this study, and low serum levels of vitamin D (22) implicate a possible role of vitamin D deficiency in MM pathogenesis. In addition, a protective effect of vitamin D (produced at least in part by sun exposure) might explain previous ambiguous results, such as the increased incidence of melanoma associated with sunscreen use (54), the protective effect of an all-year tan (55), and the lack of an association of MM with outdoor occupation (56). The effect on vitamin D status in these circumstances warrants further investigation, particularly in the climates where these associations have been reported.

REFERENCES

1. Boyle, P., Maisonneuve, P., and Dore, J-F. Epidemiology of malignant melanoma. Br. Med. Bull., *51:* 523–547, 1995.
2. Rivers, J. K. Melanoma. Lancet, *347:* 803–806, 1996.
3. Mackie, R. M., Smyth, J. F., Soutar, D. S., Watson, A. C. H., McLaren, K. M., McPhie, J. L., Hutcheon, A. W., Smyth, J. F., Calman, K. C., Hunter, J. A. A., MacGillivray, J. B., Rankin, R., and Kemp, I. W. Malignant melanoma in Scotland 1979–1983. Lancet, *2:* 859–862, 1985.
4. MacKie, R., Hunter, J. A., Aitchison, T. C., Hole, D., McLaren, K., Rankin, R., Blessing, K., Evans, A. T., Hutcheon, A. W., and Jones, D. H. Cutaneous malignant melanoma, Scotland, 1979–1989. The Scottish Melanoma Group. Lancet, *339:* 971–975, 1992.
5. Valverde, P., Healy, E., Sikkink, S., Haldane, F., Thody, A. J., Carothers, A., Jackson, I., and Rees, J. L. The Asp84glu variant of the melanocortin-1 receptor (MC1R) is associated with melanoma. Hum. Mol. Genet., *5:* 1663–1666, 1996.
6. Ichii-Jones, F., Lear, J. T., Heagerty, A. H. M., Smith, A. G., Hutchinson, P. E., Osborne, J., Bowers, B., Jones, P. W., Davies, E., Ollier, W. E. R., Thomson, W., Yengi, L., Bath, J., Fryer, A. A., and Strange, R. C. Susceptibility to melanoma: influence of skin type and polymorphism in the melanocyte stimulating hormone receptor gene. J. Investig. Dermatol., *111:* 218–221, 1998.
7. Lafuente, A., Molina, R., Palou, J., Castel, T., Moral, A., and Trias, M. Phenotype of glutathione *S*-transferase Mu (GSTM1) and susceptibility to malignant melanoma. MMM group. Multidisciplinary Malignant Melanoma Group. Br. J. Cancer, *72:* 324–326, 1995.
8. Wolf, C. R., Smith, C. A. D., Gough, A. C., Moss, J. E., Vallis, K. A., Howard, G., Carey, F. J., Mills, K., McNee, W., Carmichael, J., and Spurr, N. Relationship between the debrisoquine polymorphism and cancer susceptibility. Cancinogenesis (Lond.), *13,:* 1035–1038, 1992.
9. Strange, R. C., Ellison, T. Ichii-Jones, F., Bath, J., Hoban, P., Lear, J. T., Smith, A. G., Hutchinson, P. E., Osborne, J., Bowers, B., Jones, P. W., and Fryer, A. A. Cytochrome P450 CYP2D6 genotypes: association with hair colour, Breslow thickness and melanocyte stimulating hormone receptor alleles in patients with malignant melanoma. Pharmacogenetics, *9:* 269–276, 1999.
10. Wagner, S. N., Wagner, C., Briedigkeit, L., and Goos, M. Homozygous deletion of the $p16^{INK4a}$ and the $p15^{INK4b}$ tumour suppressor genes in a subset of human sporadic cutaneous malignant melanoma. Br. J. Dermatol., *138:* 13–21, 1998.
11. Mangelsdorf, D. J., Koeffler, H. P., Donaldson, C. A., Pike, J. W., and Haussler, M. R. 1,25-Dihydroxyvitamin D3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. J. Cell. Biol., *98:* 391–398, 1984.
12. Peehl, D. M., Skowronski, R. J., Leung, G. K., Wong, S. T., Stamey, T. A., and Feldman, D. Antiproliferative effects of 1,25-dihydroxyvitamin D3 on primary cultures of human prostatic cells. Cancer Res., *54:* 805–810, 1994.
13. Shabahang, M., Buras, R. R., Davoodi, F., Schumaker, L. M., Nauta, R. J., and Evans, S. R. 1,25-Dihydroxyvitamin D3 receptor as a marker of human colon carcinoma cell line differentiation and growth inhibition. Cancer Res., *53:* 3712–3718, 1993.
14. Frappart, L., Falette, N., Lefebvre, M. F., Bremond, A., Vauzelle, J. L., and Saez, S. *In vitro* study of effects of 1,25 dihydroxyvitamin D3 on the morphology of human breast cancer cell line BT20. Differentiation, *40:* 63–69, 1989.
15. Janowsky, E. C., Hulka, B. S., and Lester, G. E. Vitamin D levels as a risk for female breast cancer. *In:* A. W. Norman, R. Bouillon, and M. Thomasset (eds.), Vitamin D. A Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications, pp. 496–497. Berlin: de Gruyter, 1994.
16. Ingles, S. A., Haile, R., Henderson, B., Kolonel, L., and Gerhard, C. Association of vitamin D receptor genetic polymorphism with breast cancer risk in African-American and Hispanic women. *In:* A. W. Norman, R. Bouillon, and M. Thamasset (eds.), Vitamin D. Chemistry, Biology and Clinical Applications of the Steroid Hormone, pp. 813–814. University of California, Riverside, CA, 1997.
17. Corder, E. H., Guess, H. A., and Hulka, B. S. Vitamin D and prostate cancer: a prediagnostic study with stored sera. Cancer Epidemiol. Biomark. Prev., *2:* 467–472, 1993.
18. Taylor, J. A., Hirvonen, A., Watson, M., Pittman, G., Mohler, J. L., and Bell, D. A. Association of prostate cancer with vitamin D receptor gene polymorphism. Cancer Res., *56:* 4108–4110, 1996.
19. Glass, A. R., Kikendall, J. W., Sobin, L. H., and Bowen, P. E. Serum 25-hydroxyvitamin D concentrations in colonic neoplasia. Horm. Metab. Res., *25:* 397–398, 1993.

20. Colston, K., Colston, M. J., and Feldman, D. 1,25-Dihydroxyvitamin D_3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. Endocrinology, *108:* 1083–1086, 1981.

21. Ranson, M., Posen, S., and Mason, R. Human melanocytes as a target tissue for hormones: *in vitro* studies with 1α,25-dihydroxyvitamin D_3, α-melanocyte stimulating hormone, and β-estradiol. J. Investig. Dermatol., *91:* 593–598, 1988.

22. Cornwell, M. L., Comstock, G. W., Holick, M. F., and Bush, T. L. Prediagnostic serum levels of 1,25-dihydroxyvitamin D and malignant melanoma. Photodermatol. Photoimmunol. Photomed., *9:* 109–112, 1992.

23. Haussler, M. R., Jurutka, P. W., Haussler, C. A., Hsieh, J-C., Thompson, P. D., Remus, L. S., Selznick, S. H., Encinas, C., and Whitfield, G. K. VDR-mediated transactivation: interplay between $1,25(OH)_2D_3$, RXR heterodimerization, transcription (co)factors and polymorphic receptor variants. *In:* R. Norman, R. Bouillon, and M. Thomasset (eds.), Vitamin D. Chemistry, Biology and Clinical Applications of the Steroid Hormone, pp. 210–217. University of California, Riverside, CA, 1997.

24. Haussler, M. R., Whitfield, G. K., Haussler, C. A., Hsieh, J. C., Thompson, P. D., Selznick, S. H., Dominguez, C. E., and Jurutka, P. W. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J. Bone Miner. Res., *13:* 325–349, 1998.

25. Fitzpatrick, T. B. The validity and practicality of sun reaction skin types I through VI. Arch. Dermatol., *124:* 869–871, 1988.

26. Gross, C., Eccleshall, T. R., Malloy, P. J., Villa, M. L., Marcus, R., and Feldman, D. The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. J. Bone Miner. Res., *11:* 1850–1855, 1996.

27. Spector, T. D., Keen, R. W., Arden, N. K., Morrison, N. A., Major, P. J., Nguyen, T. V., Kelly, P. J., Baker, J. R., Sambrook, P. N., Lanchbury, J. S., and Eisman, J. A. Influence of vitamin D receptor genotype on bone mineral density in postmenopausal women: a twin study in Britain. Br. Med. J., *310:* 1357–1360, 1995.

28. Altman, D. G. Practical Statistics for Medical Research, pp. 241–265. London: Chapman and Hall, 1991.

29. Flanagan, L., Ethier, S., and JoEllen, W. Vitamin D-induced apoptosis in estrogen independent breast cancer cells and tumours. *In:* A. Norman, R. Bouillon, and M. Thomasset (eds.), Vitamin D. Chemistry, Biology and Clinical Applications of the Steroid Hormone, pp. 459–460. University of California, Riverside, CA, 1997.

30. Frampton, R. J., Omond, S. A., and Eisman, J. A. Inhibition of human cancer cell growth by 1,25-dihydroxyvitamin D_3 metabolites. Cancer Res., *43:* 4443–4447, 1983.

31. Skowronski, R. J., Peehl, D. M., and Feldman, D. Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D_3 receptors and actions in human prostate cancer cell lines. Endocrinology, *132:* 1952–1960, 1993.

32. Cross, H. M., Pavelka, M., Slavik, J., and Peterlik, M. Growth control of human colon cancer cells by vitamin D and calcium *in vitro*. J. Natl. Cancer Inst., *84:* 1355–1357, 1992.

33. Koeffler, H. P., Hirji, K., and Itri, L. 1,25-Dihydroxyvitamin D_3: *in vivo* and *in vitro* effects on human preleukaemic and leukaemic cells. Cancer Treat. Rep., *69:* 1399–1407, 1985.

34. Nagakura, K., Abe, E., Suda, T., Hayakawa, M., Nakamura, H., and Tazaki, H. Inhibitory effect of 1α,25-dihydroxyvitamin D3 on the growth of the renal carcinoma cell line. Kidney Int., *29:* 834–840, 1986.

35. Beaty, M. M., Lee, E. Y., and Glauert, H. P. Influence of dietary calcium and vitamin D on colon epithelial cell proliferation and 1,2-dimethylhydrazine induced colon carcinogenesis in rats fed high fat diets. J. Nutr., *123:* 144–152, 1993.

36. Chida, K., Hashiba, H., Fukushima, M., Suda, T., and Kuroki, T. Inhibition of tumour promotion in mouse skin by 1,25-dihydroxyvitamin D_3. Cancer Res., *45:* 5426–5430, 1985.

37. Ingles, S. A., Ross, R. K., Yu, M. C., Irvine, R. A., La Pera, G., Haile, R. W., and Coetzee, G. A. Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. J. Natl. Cancer Inst., *89:* 166–170, 1997.

38. Milde, P., Hauser, U., Simon, T., Mall, G., Ernst, V., Haussler, M., Frosch, P., and Rauterberg, W. Expression of 1,25-dihydroxyvitamin D3 receptors in normal and psoriatic skin. J. Investig. Dermatol., *97:* 230–239, 1991.

39. Ingles, S. A., Haile, R. W., Henderson, B. E., Kolonel, L. N., Nakaichi, G., Shi, C. Y., Yu, M. C., Ross, R. K., and Coetzee, G. A. Strength of linkage disequilibrium between two vitamin D receptor markers in five ethnic groups: implications for association studies. Cancer Epidemiol. Biomark. Prev., *6:* 93–98, 1997.

40. Reintgen, D. S., Cox, C., Slingluff, C. L. J., and Seigler, H. F. Recurrent malignant melanoma: the identification of prognostic factors to predict survival. Ann. Plast. Surg., *28:* 45–49, 1992.

41. Miyamoto, K., Kesterson, R. A., Yamamoto, H., Taketani, Y., Nishiwaki, E., Tatsumi, S., Inoue, Y., Morita, K., Takeda, E., and Pike, J. W. Structural organisation of the human vitamin D receptor chromosomal gene and its promoter. Mol. Endocrinol., *11:* 1165–1179, 1997.

42. Liu, M., Lee, M. H., Bommakanti, M., and Feedman, L. P. Vitamin D_3 transcriptionally activates the *p21* gene leading to the induced differentiation of the myelomonocytic cell line U937. Genes Dev., *10:* 142–152, 1996.

43. Liu, M., Lee, M. H., Cohen, M., Bommakanti, M., and Freedman, L. P. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. Genes Dev., *10:* 142–153, 1996.

44. Wang, Q. M., Jones, J. B., and Studzinski, G. P. Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D_3 in HL60 cells. Cancer Res., *56:* 264–267, 1996.

45. Verlinden, L., Verstuyf, A., Convents, R., Marcelis, S., Van Camp, M., and Bouillon, R. Action of $1,25(OH)_2D_3$ on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. Mol. Cell. Endocrinol., *142:* 57–65, 1998.

46. Piccinin, S., Doglioni, C., Maestro, R., Vukosavljevic, T., Gasparotto, D., D'Orazi, C., and Boiocchi, M. p16/CDKN2, and CDK4 gene mutations in sporadic melanoma development and progression. Int. J. Cancer, *74:* 26–30, 1997.

47. Ohta, M., Berd, D., Shimizu, M., Nagai, H., Cotticelli, M. G., Mastrangelo, M., Shields, J. A., Shields, C. L., Croce, C. M., and Huebner, K. Deletion mapping of chromosome region 9p21–p22 surrounding the *CDKN2* locus in melanoma. Int. J. Cancer, *65:* 762–767, 1996.

48. Platz, A., Hansson, J., Mansson-Brahme, E., Lagerlof, B., Linder, S., Lundqvist, E., Sevigny, P., Inganas, M., and Ringborg, U. Screening of germline mutations in the *CDKN2A* and *CDKN2B* genes in Swedish families with hereditary cutaneous melanoma. J. Natl. Cancer Inst., *89:* 697–702, 1997.

49. Soufir, N., Avril, M. F., Chompret, A., Demenais, F., Bombled, J., Spatz, A., Stoppa-Lyonnet, D., Benard, J., and Bressac-de Paillerets, B. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. Hum. Mol. Genet., *7:* 209–216, 1998.

50. Tsao, H., Benoit, E., Sober, A. J., Thiele, C., and Haluska, F. G. Novel mutations in the p16/CDKN2A binding region of the *cyclin-dependent kinase-4* gene. Cancer Res., *58:* 109–113, 1998.

51. Garland, F. C., Garland, C. F., Gorham, E. D., and Young, J. F. Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. Prev. Med., *19:* 614–622, 1990.

52. Hanchette, C. L., and Schwartz, G. G. Geographic patterns of prostate cancer mortality. Cancer (Phila.), *70:* 2861–2869, 1992.

53. Gorham, E. D., Garland, C. F., and Garland, F. C. Acid haze air pollution and breast and colon cancer mortality in 20 Canadian cities. Can. J. Public Health, *80:* 96–100, 1989.

54. Autier, P., Dore, J. F., Schifflers, E., Cesarini, J. P., Bollaerts, A., Koelmel, K. F., Gefeller, O., Liabeuf, A., Lejeune, F., and Lienard, D. Melanoma and use of sunscreens: an EORTC case-control study in Germany, Belgium, and France. The EORTC Melanoma Cooperative Group. Int. J. Cancer, *61:* 749–755, 1995.

55. Holly, E. A., Aston, D. A., Cress, R. D., Ahn, D. K., and Kristiansen, J. J. Cutaneous melanoma in women. Am. J. Epidemiol., *141:* 923–933, 1995.

56. Pion, I. A., Rigel, D. S., Garfinkel, L., Silverman, M. K., and Kopf, A. W. Occupation and the risk of malignant melanoma. Cancer (Phila.), *75:* 637–644, 1995.