EUROPEAN JOURNAL OF CANCER 45 (2009) 3271–3281

available at www.sciencedirect.com

journal homepage: www.ejconline.com

Vitamin D receptor gene polymorphisms, serum 25-hydroxyvitamin D levels, and melanoma: UK case–control comparisons and a meta-analysis of published *VDR* data

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ARTICLE INFO

Article history:
Received 1 April 2009
Received in revised form 28 May 2009
Accepted 10 June 2009
Available online 15 July 2009

Keywords:
Vitamin D
Breslow thickness
Heredity
Body mass index

ABSTRACT

We have carried out melanoma case–control comparisons for six vitamin D receptor (*VDR*) gene single nucleotide polymorphisms (SNPs) and serum 25-hydroxyvitamin D_3 levels in order to investigate the role of vitamin D in melanoma susceptibility. There was no significant evidence of an association between any *VDR* SNP and risk in 1028 population-ascertained cases and 402 controls from Leeds, UK. In a second Leeds case–control study (299 cases and 560 controls) the FokI T allele was associated with increased melanoma risk (odds ratio (OR) 1.42, 95% confidence interval (CI) 1.06–1.91, $p = 0.02$). In a meta-analysis in conjunction with published data from other smaller data sets (total 3769 cases and 3636 controls), the FokI T allele was associated with increased melanoma risk (OR 1.19, 95% CI 1.05–1.35), and the BsmI A allele was associated with a reduced risk (OR 0.81, 95% CI 0.72–0.92), in each instance under a parsimonious dominant model. In the first Leeds case–control comparison cases were more likely to have a higher body mass index (BMI) than controls ($p = 0.007$ for linear trend). There was no evidence of a case–control difference in serum 25-hydroxyvitamin D_3 levels. In 1043 incident cases from the first Leeds case–control study, a single estimation of serum 25-hydroxyvitamin D_3 level taken at recruitment was inversely correlated with Breslow thickness ($p = 0.03$ for linear trend). These data provide evidence to support the view that vitamin D and *VDR* may have a small but potentially important role in melanoma susceptibility, and putatively a greater role in disease progression.

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.06.011

1. Introduction

High penetrance genes such as *CDKN2A* and *CDK4* underlie susceptibility to melanoma in rare families.[1] A number of phenotypic factors that are in part genetically determined, including number of common and atypical nevi, red or blonde hair, skin type and tanning ability, have been identified as risk factors for melanoma in the general population.[2] Recent large-scale association studies have shown that the genetic determinants of some of these phenotypes, such as red hair and freckles,[3] are also risk factors for melanoma.[4,5] These genes include the melanocortin 1 receptor (*MC1R*) gene, which was previously identified as a melanoma susceptibility gene in case–control association and familial melanoma studies.[5–9] The large-scale association studies have also provided strong evidence to support the role of other pigment genes such as the agouti signalling protein (*ASIP*) locus and tyrosinase (*TYR*)[4,10] as melanoma susceptibility genes. There are some reports of other associations with candidate genes and risk but the studies are small and sometimes conflicting.

25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of the steroid hormone vitamin D_3, is a potent regulator of cell growth and differentiation[11] and moderates gene transcription by binding to the vitamin D receptor (VDR). 25-Hydroxyvitamin D_3 is also implicated in cell death, tumour invasion and angiogenesis,[11,12] making it an important candidate for moderating both risk of melanoma and prognosis from melanoma. In this study we have looked at inheritance of variants in the *VDR* gene and susceptibility to sporadic melanoma.

Five *VDR* polymorphisms (Cdx2, rs11568820; FokI, rs2228570, previously known as rs10735810; BsmI, rs1544410; ApaI, rs7975232 and TaqI, rs731236), primarily chosen based on predicted changes in receptor activity and/or expression based on *in vitro* and *in vivo* evidence reviewed by Uitterlinden,[13] have been studied by other groups. The functional consequences of the Cdx2 and FokI variants have been extensively characterised, whilst the functionality of the common BsmI–ApaI–TaqI haplotypes is less clear, despite the publication of a very large number of studies looking at the association of these haplotypes with many different diseases. More recently, an additional promoter polymorphism has been described in a melanoma population, 1012 bp upstream of the exon 1a transcription start site (A-1012G, also known as GATA; rs4516035).[14]

Previous studies have reported associations between some or all of these *VDR* polymorphisms and risk and/or outcome of a number of malignancies, including prostate, colon, breast and renal cancers.[15–19] However, there have been a limited number of small studies examining the role of *VDR* polymorphisms in melanoma.[14,20–24] In 2008, one of these studies was updated in the literature, with increased numbers of cases, and inclusion of BsmI for the first time.[25] Recently two meta-analyses were reported that support the view that BsmI[26] and both BsmI and FokI,[27] respectively, are associated with melanoma risk. Here, we looked at these six single nucleotide polymorphisms (SNPs) in two Leeds case–control data sets to investigate the effect on risk of melanoma and on Breslow thickness.

Sunburn and intense intermittent sun exposure are associated with an increased risk of melanoma.[28,29] The lack of risk for most body sites associated with occupational sun exposure at most latitudes however has led to the view that sun exposure has a complex relationship with melanoma risk, and that chronic sun exposure might even be protective for melanoma in some low sun exposure countries as a result of enhanced vitamin D synthesis in the skin.[28] Clarification of the potential association of vitamin D and the *VDR* with melanoma risk remains therefore of importance.

Vitamin D levels are known to be lower in obese subjects,[30–32] and we have therefore also explored the relationship between body mass index (BMI), serum 25-hydroxyvitamin D_3 levels (henceforth referred to as serum vitamin D levels) and melanoma risk. Increased BMI has been reported to be associated with melanoma risk. The observation was first made by Thune et al.[33] and subsequently by a series of other groups.[34–38] The underlying biological explanation for this association is not understood, although one study provided some evidence that high fasting glucose was associated with melanoma risk.[39]

In two Leeds case–control series, we have looked at inheritance of the aforementioned six single nucleotide polymorphisms (SNPs) in *VDR* to explore susceptibility to sporadic melanoma. We carried out a meta-analysis of all published SNP data including our new Leeds data. We looked at the effect of these SNPs and of serum vitamin D levels taken at study recruitment on Breslow thickness as a marker of tumour progression.

2. Materials and methods

2.1. The first Leeds case–control series (Leeds CCS1)

Population-ascertained incident melanoma cases have been recruited to a case–control study since 2000 in a geographically defined area of Yorkshire and the northern region of the UK (67% participation rate). Thousand and forty-three male and female patients (aged between 18 and 75 years) were diagnosed in the period from September 2000 to December 2006. The cases were identified via clinicians, pathology registers and the cancer registry to ensure maximal ascertainment. Between September 2000 and June 2003 all patients with invasive melanoma were invited to participate. From July 2003 till December 2006, patients with Breslow thickness less than 0.75 mm were not invited as the cases were also being recruited as a cohort looking at prognostic outcomes. The 408 population-ascertained controls were identified from the cases' family doctors (55% response rate), and one control was matched to 1 or more cases by age and sex. A small number of samples were dropped due to incomplete genotyping: descriptive statistics of cases and controls with complete data sets are given in Supplementary material (Table 1s).

The following data were available from participants recruited to this study. Age, sex, Townsend score[40] (a deprivation measure derived from postcode), self-reported natural hair colour at age 18, skin type and height and weight (for body mass index (BMI) and body surface area (BSA) calculation) were recorded at interview. Breslow thickness of the pri-

mary tumour was obtained from histology reports. DNA was extracted from blood from consenting participants to allow detection of *VDR* polymorphisms. Serum was cryopreserved at recruitment from cases and a proportion of controls for the measurement of serum vitamin D levels.

2.2. The second Leeds case series (Leeds CCS2)

A further 300 melanoma patients (66% participation rate) of both sexes aged over 18 years, who were recruited into a study of late relapse in melanoma, were also studied. One sample was excluded because of failure to genotype. Half of the cases had relapsed from their melanoma and all were at least three years post diagnosis. Recruitment of patients took place between May 2000 and January 2005 from geographically defined areas of the UK (the Northern and Yorkshire, Oxford, Trent and West Midlands Health Regions). This study is described fully elsewhere in a paper in which questionnaire data collected from 274 of the 300 cases were reported.[41]

2.3. The second Leeds control series (Leeds CCS2)

The comparison group for the second case series comprises 697 unselected female healthy controls aged 19–46 years who were recruited via two groups of family doctors from Yorkshire ($n = 396$) and another group working in an area of the UK approximately 200 miles south of Yorkshire ($n = 301$).[42] All women in the age group who were registered with the doctors were invited to participate, and the study is described in full elsewhere.[43] In total, 572 women agreed to give blood for extraction of germline DNA (82%). These women were recruited as volunteers in a study examining the relationship between ultra-violet (UV) radiation exposure and sunbed usage in determining patterns of skin naevi and factors associated with skin aging.

Age and sex were recorded for participants in this study, and DNA was extracted from blood from consenting participants to allow detection of *VDR* polymorphisms.

All participating individuals, regardless of study, gave written informed consent, and regional ethical committee approval was obtained.

2.4. SNP methodology

The Cdx2, GATA, FokI and ApaI SNPs were amplified using allele-specific differentially labelled fluorescent polymerase chain reaction (PCR). The BsmI and TaqI SNPs were examined using restriction enzyme (New England Biolabs, Hitchin, UK) digest of differentially labelled fluorescent PCR products. Further information on the SNPs, including oligonucleotide sequences, is provided as Supplementary information (Table 2s). Amplification was performed using Hotstar Mastermix (Qiagen, UK) according to supplied protocols with an annealing temperature of 52 °C in three multiplex reactions. All products were detected using a 3130XL ABI Automated Sequencer (Applied Biosystems, Warrington, UK) and analysed using GeneMarker v1.6 genotyping software (Softgenetics, Pennsylvania, US).

2.5. Serum collection

Serum from all cases was aliquoted and frozen on arrival at the laboratory in Leeds at –80 °C. Patients were recruited where possible in the period 3–6 months after diagnosis, although sometimes later if patients responded slowly to the invitation to participate. Twenty-eight patients presented with metastasis at diagnosis or developed recurrence very quickly so that serum samples were obtained in the presence of metastatic disease; these samples were excluded from the analysis. Serum samples were also obtained from population controls recruited to Leeds CCS1 between October 2002 and December 2005 and then again between May 2007 and May 2008, so that we did not have serum vitamin D level results for all subjects. There was no difference however in the serum levels of vitamin D in controls collected during these two time periods (data not shown).

The serum samples were mailed to the laboratory, and therefore there was variation in the time taken to freezing. The protocol asked that samples be frozen as near to 48 h after venepuncture as possible to ensure uniformity across the study. The time to freezing was recorded to allow variation occurring as a result of this to be quantified, although previous experiments in the laboratory have shown that serum 25-hydroxyvitamin D_3 levels are stable over short time periods on the bench and if cryopreserved, at –80 °C ($n = 243$, Spearman correlation for serum vitamin D levels and time to processing, $r_S = 0.01$, $p = 0.83$). In the Leeds CCS1 68% of the samples were frozen within 24 h of venepuncture, 20% within 48 h, 9% within 72 h and 3% longer than 72 h. Again, there was no relationship between serum 25-hydroxyvitamin D_3 levels and time to freezing of the sample ($r_S = 0.05$, $p = 0.22$). Vitamin D levels fluctuate with season. The samples were taken throughout the year, and the results were therefore adjusted for month of venepuncture in the analysis to correct for this.

2.6. Measurement of serum 25-hydroxyvitamin D_2 and D_3

Assays were carried out in the Leeds Teaching Hospital's NHS Biochemistry laboratory. Deuterated 25-OH vitamin D_3 was used as internal standard. The Waters Quattro Micro MS/MS was operated in multiple-reaction monitoring mode with the transitions $m/z + 413.2 > 82.7$ for 25-hydroxyvitamin D_2, $m/z + 401.2 > 159.1$ for 25-OH vitamin D_3 and $m/z + 407.2 > 159.1$ for hexadeuterated 25-hydroxyvitamin D_3. Calibrators were prepared by spiking 25-hydroxyvitamin D_2 and D_3 into vitamin D-depleted serum. Pools of serum samples were also spiked with 25-hydroxyvitamin D_2 or D_3 as was necessary to prepare quality control samples. The working range of the assay is 10–250 nmol/l. Interassay imprecision for both metabolites was less than 10% at concentrations ranging from 12–159 nmol/l. Recovery of added 25-hydroxyvitamin D_2 and D_3 into pooled samples ranged from 85% to 116%. Results for 25-hydroxyvitamin D_2 and D_3 were pooled.

2.7. Statistical methods

Exact Hardy Weinberg equilibrium tests were performed among the combined control subjects for each of the SNPs.

Logistic regression was used to estimate the crude odds ratio (OR) (with 95% confidence interval (CI)) for melanoma for each genotype, compared with common homozygotes as baseline, for each SNP independently. A Cochran–Armitage trend test was also performed for each SNP. Analysis of variance (ANOVA) was used to test differences in Breslow thickness (log-transformed) between genotypes of each SNP.

Original articles reporting on an association between *VDR* polymorphisms and melanoma risk published up to May 2009 were identified using a systematic search of the PubMed, Medline, Cancerlit and ISI Web of Knowledge databases. Keywords used to perform the search were (in various combinations): 'melanoma', 'risk', 'vitamin D receptor', '*VDR*', 'polymorphism', 'gene', 'allele' and 'Breslow thickness'. Where more than one publication from a single group was identified that reported on the same case series and polymorphisms, only the most recent publication was considered. We included both Leeds CCS1 and Leeds CCS2 in the meta-analysis and sensitivity analyses were performed.

Meta-analyses were conducted by fitting random effects models and were checked for small size and publication bias using funnel plots.[44] Separate pair-wise comparisons for heterozygotes and homozygotes using a common baseline were performed using a bivariate approach to account for the correlation between the two risk estimates. Heterogeneity was investigated using I^2, which describes the proportion of total variation in study estimates that is due to heterogeneity and by the χ^2 statistic. All analyses were performed using STATA software version 10 (StataCorp, College Station, TX, USA), including the 'metan' and 'hapipf' STATA modules for meta-analysis and haplotype analysis, respectively.

Serum vitamin D levels (based on a single serum sample taken at recruitment to the study, shortly after diagnosis for cases) were shown to be normally distributed (Shapiro-Wilk statistic $W = 0.976$), and were assessed as a continuous variable in the analyses. Spearman correlation (r_S) was used to assess correlations between serum vitamin D levels and time to processing, BMI and Townsend score. Adjusted means (least squares means) of serum vitamin D levels corrected for sex, age and month sampled were calculated for different BMI and Breslow thickness groups. BMI was grouped according to the World Health Organisation classification where <24.9 is classified as normal, >24.9 but <29.9 as overweight and >29.9 as obese. BSA was grouped as <1.81, 1.81–2.03 and >2.04 m^2 based on the DuBois formula. Logistic regression models adjusted for age, sex, BMI and deprivation score (Townsend Score based on 2001 UK Census data[40]) were used to examine the effects of serum vitamin D levels (stratified by quarter of the year to allow for seasonal variation) on risk of melanoma. The logistic regression model can be written as

$$\mathrm{logit}(p_i) = \mathbf{x}_i'\beta + z_1\gamma_1 + z_2\gamma_2 + z_3\gamma_3 + z_4\gamma_4,$$

where $\mathbf{x}_i$ is a vector including confounding variables and z_i is the measured serum vitamin D levels in season i of venepuncture (i = 1–4); β, γ_1, γ_2, γ_4} are the regression coefficients. If a participant had blood taken in the first season, then $z_2 = z_3 = z_4 = 0$. These analyses were carried out using GLM and LOGISTIC procedures in the SAS software version 9.1 for PC (Copyright, SAS Institute Inc., Cary, NC, USA).

3. Results

The proportion of missing data per SNP in the Leeds studies overall (due to failure to genotype) ranged from 0.3% for Cdx2 to 0.7% for FokI. In total, 34 individuals (16 cases, 18 controls) were excluded from the analyses because of incomplete genotype data, leaving a total of 2289 subjects (1327 cases, 962 controls in the two Leeds studies).

3.1. *Leeds CCS1*

Table 1 shows the frequency of the six *VDR* SNPs in the 1028 population-ascertained incident cases and 402 controls in Leeds CCS1. Among controls, the genotype distribution did not deviate from Hardy Weinberg equilibrium (Table 1). There was no significant difference in genotype distribution between cases and controls. The ORs and 95% CIs are also presented in Table 1.

The Cdx2 and GATA polymorphisms were found to be in strong linkage disequilibrium (LD) ($D' = 0.97$), with three common haplotypes (G–G 42%, G–A 36% and A–A 21% for the Cdx2 and GATA variants, respectively) in Leeds CCS1. There was no evidence of association with melanoma status for any haplotype (data not shown). The BsmI, ApaI and TaqI polymorphisms were also in strong LD as previously reported ($D' = 0.97$), with three haplotypes accounting for 97% of all haplotypes (G–C–T 45%, A–A–C 40%, G–A–T 12% for the BsmI–ApaI–TaqI haplotype). There was no evidence of any association between any haplotype and melanoma status (data not shown). The FokI SNP was not found to be in LD with any of the other SNPs analysed.

The mean deprivation scores were –0.06 (standard deviation (SD) 3.03) and –0.23 (SD 2.95) for non-responding and responding controls, respectively, which were not significantly different ($p = 0.41$). Cases who declined to participate in the study were more deprived than those consented into the study ($p = 0.001$), the average deprivation scores being 0.79 (SD 3.33) and 0.22 (SD 3.10) for the non-participating and participating cases. Of those who participated in the study, melanoma cases were more deprived than controls ($p = 0.007$). Density plots of deprivation scores of participating and non-participating cases and controls are given in Fig. 1s. The distribution of scores is skewed with a tail of individuals with postcodes indicative of greater deprivation.

Negative correlations were observed between BMI and serum vitamin D levels in Leeds CCS1 (Spearman $r_S = -0.11$, $p = 0.002$ for cases, and $r_S = -0.26$, $p = 0.005$ for controls). Lower vitamin D levels were slightly more common in those with higher deprivation scores, with Spearman correlations of –0.10 ($p = 0.003$) and –0.17 ($p = 0.09$) for cases and controls, respectively. Higher deprivation scores were associated with increased BMI in both cases and controls, with Spearman correlations of 0.08 ($p = 0.01$) and 0.11 ($p = 0.03$), respectively.

As serum vitamin D levels vary with BMI, BMI was assessed at recruitment for individuals in Leeds CCS1. Cases had a significantly higher BMI than controls. Relative to a BMI of <24.9 kg/m^2, the ORs for developing a melanoma among people with BMI 24.9–29.9 and BMI >29.9, adjusting

Table 1 – A case–control comparison of risks of melanoma in the first Leeds case–control series in relation to VDR SNP genotypes.

SNP	Genotype	Cases (n = 1028)	Controls (n = 402)	Population ascertained melanoma cases (n = 1028), and controls (n = 402)			
				OR	95% CI	p^*	Trend p^a
Cdx2	GG	648 (63.0)	250 (62.2)	1			
	GA	324 (31.5)	134 (33.3)	0.93	(0.73, 1.20)	0.65	0.97
	AA	56 (5.5)	18 (4.5)	1.20	(0.69, 2.08)		
GATA	AA	337 (32.8)	137 (34.1)	1			
	AG	509 (49.5)	188 (46.8)	1.10	(0.85, 1.43)	0.63	0.97
	GG	182 (17.7)	77 (19.2)	0.96	(0.69, 1.34)		
FokI	CC (FF)	381 (37.1)	161 (40.1)	1			
	CT (Ff)	489 (47.6)	176 (43.8)	1.17	(0.91, 1.51)	0.43	0.59
	TT (ff)	158 (15.4)	65 (16.2)	1.03	(0.73, 1.47)		
BsmI	GG (bb)	356 (34.6)	134 (33.3)	1			
	GA (Bb)	497 (48.4)	202 (50.3)	0.93	(0.72, 1.20)	0.81	0.87
	AA (BB)	175 (17.0)	66 (16.4)	1.00	(0.71, 1.41)		
ApaI	CC (aa)	221 (21.5)	92 (22.9)	1			
	AC (Aa)	524 (51.0)	190 (47.3)	1.15	(0.86, 1.54)	0.45	0.82
	AA (AA)	283 (27.5)	120 (29.9)	0.98	(0.71, 1.36)		
TaqI	TT (TT)	369 (35.9)	144 (35.8)	1			
	TC (Tt)	484 (47.1)	194 (48.3)	0.97	(0.75, 1.26)	0.86	0.80
	CC (tt)	175 (17.0)	64 (15.9)	1.07	(0.76, 1.51)		

* p-Values from models comparing genotype frequencies (2 degrees of freedom).
a p-Values from Cochran–Armitage trend test.

for age, sex, BSA and deprivation score, were, respectively, 1.48 (95% CI 1.11–1.98) and 1.78 (95% CI 1.17–2.71) (p value for linear trend = 0.007). The adjusted ORs for BSA being 1.81–2.03 m^2 and >2.04 m^2 compared to BSA <1.81 m^2 were 0.87 (95% CI 0.61–1.22) and 1.05 (95% CI 0.65–1.71), respectively. BMI but not BSA was associated with melanoma risk in the multivariable analysis.

Serum vitamin D levels were compared for the 941 cases and 114 controls from Leeds CCS1 for whom we had full data sets, stratified by season of sampling to allow for variation observed in levels through the year. In the 941 cases serum levels did vary, as expected, with phenotype (p=0.05) so that the mean level (adjusted for sex, age, month sampled and BMI) in 104 red-heads was 50.5 nmol/l (95% CI 46.6–54.4), in 167 blonds was 56.1 (95% CI 53.0–59.1) and in 541 with brown or black hair was 52.4 (95% CI 50.7–54.1). The crude OR for melanoma per 20 nmol/l increase in serum vitamin D ranged from 0.87 (95% CI 0.70–1.09) between October and December to 1.00 (95% CI 0.78–1.27) between April and June. There was no association between case status and serum vitamin D level at recruitment when adjusted for age, sex, deprivation score and season of sampling. The adjusted OR per 20 nmol/l increase in serum vitamin D across seasons was 0.94 (95% CI 0.79–1.12). Adjusting for BMI had no effect on this relationship. Additionally, there was no evidence of interaction between BMI and serum vitamin D levels (p = 0.21).

Breslow thickness data can be considered as a measure of tumour progression and therefore a marker of the effect of *VDR* on prognosis. Data on Breslow thickness were available for 97.4% of the cases from Leeds CCS1. Absence of data was due either to the manner of surgery (such as curettage) or to the presence of an occult primary. There was no significant association between *VDR* SNP genotype and Breslow thickness (Table 3s), but serum vitamin D levels were inversely correlated with thickness as described below.

The serum vitamin D level at recruitment was negatively correlated with tumour Breslow thickness (Table 2), test for trend p = 0.03, so that thinner tumours were associated with higher serum levels adjusting for age, sex, month of venepuncture, deprivation score and BMI, as has recently been reported in a study of survival in this cohort.[45]

3.2. *Leeds CCS2*

In the second Leeds case–control series, the genotype distribution for the controls did not deviate from Hardy Weinberg equilibrium (Table 3). In comparing the cases with the controls, there was a significant association between melanoma and the variant T allele of FokI (OR 1.28, 95% CI 0.93–1.75 for CT compared with CC, and OR 1.88, 95% CI 1.25–2.82 for TT compared with CC, p-value for difference in genotype frequencies 0.01, Table 3). If a dominant model is assumed, the FokI T allele was associated with an increased risk of melanoma, with an OR of 1.42 (95% CI 1.06–1.91), p = 0.02. The association between melanoma and the T allele did not differ for the melanoma cases who had relapsed from the melanoma cases from those who had not relapsed. Although Leeds Control 2 was an all female group, we showed that the allele frequency in these controls did not differ from the allele frequency in the population controls from Leeds CCS1 (Table 3).

Table 2 – The relationship between serum 25-dihydroxyvitamin D_2/D_3 level at recruitment and Breslow thickness in 819 cases recruited to the first Leeds case–control series. Data are adjusted for age, sex, month of blood taken, BMI and deprivation score. p-Value for trend was 0.03.

Breslow thickness	N	Crude mean (95% CI)	Adjusted mean (95% CI)
<0.75 mm	145	56.7 (52.9, 60.4)	56.0 (52.6, 59.3)
0.75–1 mm	197	54.6 (51.4, 57.7)	54.9 (52.0, 57.8)
1–2 mm	262	52.7 (50.3, 55.2)	53.6 (51.1, 56.1)
2–3 mm	105	53.0 (48.5, 57.4)	51.6 (47.6, 55.6)
>3 mm	110	49.9 (45.6, 54.2)	49.4 (45.6, 53.2)

Table 3 – A case–control comparison of risks of melanoma in the second Leeds case and control series (Leeds case 2 and Leeds control 2) in relation to VDR SNP genotypes (as the Leeds control 2 was a female group we have shown the population controls for comparison (Leeds control 1).

SNP	Genotype	Leeds case 2 (n = 299)	Leeds control 1 (n = 402)	Leeds control 2 (n = 560)	Leeds case 2 versus Leeds control 2			Leeds case 2 versus (Leeds control 1 + Leeds control 2)		
					OR (95% CI)	p^*	Trend P[a]	OR (95% CI)	p^*	Trend p[a]
Cdx2	GG	193 (64.6)	250 (62.2)	350 (62.5)	1			1		
	GA	89 (29.8)	134 (33.3)	179 (32.0)	0.90 (0.66, 1.23)	0.80	0.66	0.88 (0.66, 1.18)	0.64	0.69
	AA	17 (5.7)	18 (4.5)	31 (5.5)	0.99 (0.54, 1.84)			1.08 (0.61, 1.92)		
GATA	AA	87 (29.1)	137 (34.1)	198 (35.4)	1			1		
	AG	151 (50.5)	188 (46.8)	261 (46.6)	1.32 (0.95, 1.82)	0.17	0.09	1.29 (0.96, 1.75)	0.18	0.11
	GG	61 (20.4)	77 (19.2)	101 (18.0)	1.37 (0.92, 2.06)			1.32 (0.91, 1.92)		
FokI	CC (FF)	96 (32.1)	161 (40.1)	225 (40.2)	1			1		
	CT (Ff)	139 (46.5)	176 (43.8)	255 (45.5)	1.28 (0.93, 1.75)	0.01	0.003	1.30 (0.97, 1.74)	0.009	0.002
	TT (ff)	64 (21.4)	65 (16.2)	80 (14.3)	1.88 (1.25, 2.82)			1.77 (1.23, 2.57)		
BsmI	GG (bb)	110 (36.8)	134 (33.3)	175 (32.3)	1			1		
	GA (Bb)	145 (48.5)	202 (50.3)	284 (50.7)	0.81 (0.59, 1.11)	0.20	0.07	0.84 (0.63, 1.12)	0.27	0.11
	AA (BB)	44 (14.7)	66 (16.4)	101 (18.0)	0.69 (0.45, 1.06)			0.74 (0.50, 1.10)		
ApaI	CC (aa)	68 (22.7)	92 (22.9)	102 (18.2)	1			1		
	CA (Aa)	151 (50.5)	190 (47.3)	283 (50.5)	0.80 (0.56, 1.15)	0.19	0.07	0.91 (0.65, 1.27)	0.37	0.17
	AA (AA)	80 (26.8)	120 (29.9)	175 (31.3)	0.69 (0.46, 1.03)			0.77 (0.53, 1.12)		
TaqI	TT (TT)	107 (35.8)	144 (35.8)	187 (33.4)	1			1		
	TC (Tt)	150 (50.2)	194 (48.3)	273 (48.8)	0.96 (0.70, 1.31)	0.34	0.21	0.99 (0.75, 1.32)	0.46	0.34
	CC (tt)	42 (14.1)	64 (15.9)	100 (17.9)	0.73 (0.48, 1.13)			0.79 (0.53, 1.19)		

a p-Values from Cochran–Armitage trend test. p-Values from models comparing genotype frequencies (2 degrees of freedom).

3.3. *Meta-analysis of VDR SNPs*

A comparison of Leeds CCS1 with the genotype frequencies published in previous studies is given in Table 4s in Supplementary information. Within control populations there was no difference in allele frequencies among studies, except for BsmI ($p = 0.02$) where the Santonocito et al.'s study had higher minor allele frequency. A meta-analysis was performed for five of the six SNPs examined in this study where additional data from previous studies were available (Table 4). Looking at individual SNP genotypes, pooled ORs were estimated for each genotype compared with common homozygotes as baseline, and the FokI polymorphism (T allele) showed a significant association with melanoma (Fig. 1). There was no evidence of heterogeneity between risk estimates from different studies for CT versus baseline ($p = 0.50$, $I^2 = 0\%$) but there was some evidence of heterogeneity for TT versus baseline ($p = 0.062$, $I^2 = 50\%$). The risk estimates for FokI are consistent with a dominant effect of the T allele on risk, so an estimate of the effect assuming a dominant model was also obtained (pooled OR 1.19, 95% CI 1.05–1.35 for carriage of the T allele, $p = 0.01$, with no evidence of heterogeneity, $p = 0.29$, $I^2 = 18\%$). Funnel plots for the risk estimates for FokI genotypes showed no clear evidence of publication bias.

From the meta-analysis there was also some evidence of a protective effect on melanoma of the BsmI A allele (Table 4). The data were consistent with a dominant protective effect, and under this model the estimated pooled OR was 0.81 (95% CI 0.72–0.92). There was no evidence for heterogeneity in estimates among studies ($p = 0.34$, $I^2 = 0\%$, Fig. 2). A suggestion of a similar protective effect was observed in the second Leeds case–control series (Table 3) although the association did not reach statistical significance ($p = 0.20$).

Although some association was also seen with TaqI in the meta-analysis, there was evidence of heterogeneity between

Table 4 – Meta-analysis of current and published data on risk of melanoma.

SNP	Number of studies	Reference	Heterozygotes	Homozygotes	Heterozygotes and homozygotes
			Pooled OR (95% CI) [I^2]	Pooled OR (95% CI) [I^2]	Pooled OR (95% CI) [I^2]
Cdx-2	3	GG	0.95 (0.81, 1.12) [0%]	1.00 (0.69, 1.46) [0%]	0.96 (0.82, 1.12) [0%]
GATA	5	AA	1.09 (0.88, 1.35) [0%]	0.92 (0.62, 1.36) [63%]	1.06 (0.86, 1.30) [44%]
FokI	7	CC (FF)	1.20 (1.06, 1.35) [0%]	1.21 (0.94, 1.57) [50%]	1.19 (1.05, 1.35) [18%]
BsmI	5	GG (bb)	0.82 (0.72, 0.93) [0%]	0.79 (0.66, 0.95) [42%]	0.81 (0.72, 0.92) [0%]
TaqI	5	TT (TT)	0.96 (0.75, 1.22) [65%]	0.91 (0.69, 1.20) [46%]	0.95 (0.75, 1.20) [69%]

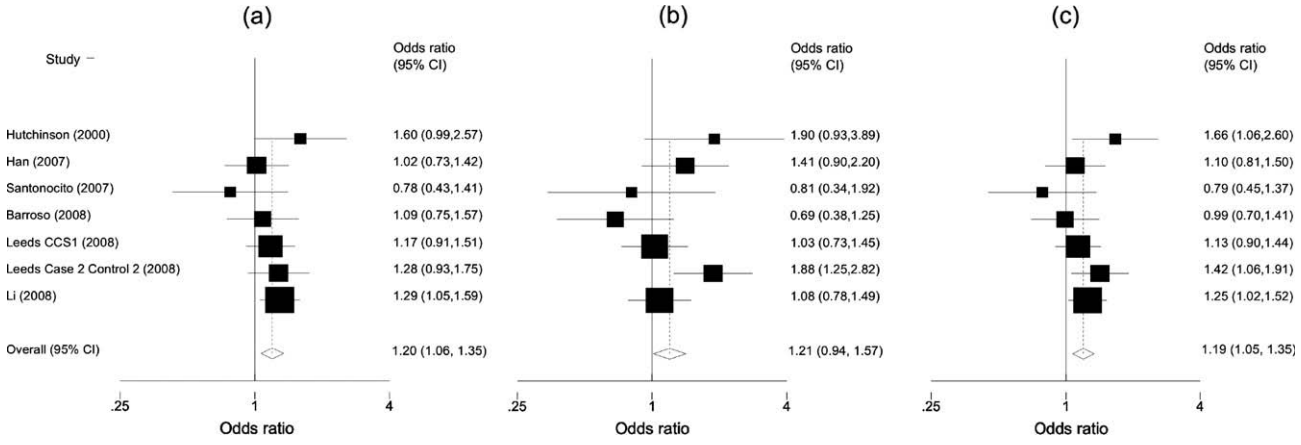

Fig. 1 – Meta-analysis of the FokI genotypes. *In vitro* studies have provided evidence that the wild type genotype is associated with greater transcription of *VDR* responsive genes than variant genotypes (CT or TT). The meta-analysis suggests that the variant allele associated with reduced transcription is associated with increased risk of melanoma; (a) compares the CT heterozygotes with the CC homozygotes (wild type); (b) compares the TT homozygotes with the CC homozygotes; (c) compares either CT or TT with the CC homozygotes.

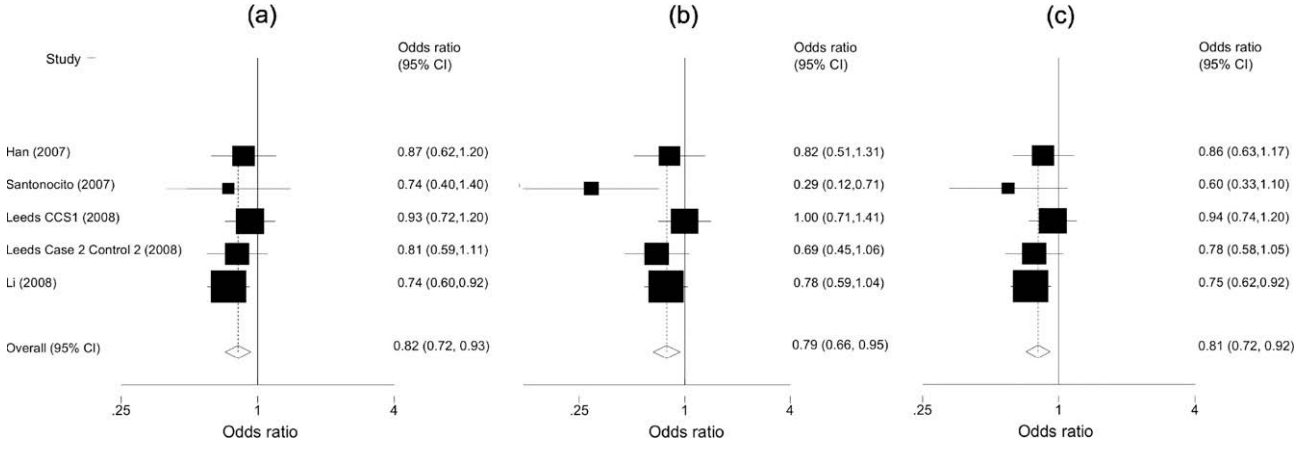

Fig. 2 – Meta-analysis of BsmI. BsmI variants are in LD with ApaI and TaqI. The biological implications of BsmI polymorphisms are less well understood than for FokI variants, but the data provide reasonably consistent evidence that the A (variant) allele is associated with lower RNA stability, yet in this meta-analysis the suggestion is that the A allele is weakly protective for melanoma. (a) GA heterozygotes compared to a baseline GG genotype (wild type); (b) AA homozygotes compared to the GG genotype; (c) compares either GA or AA with the GG homozygotes.

estimates from different studies ($p = 0.022$, $I^2 = 65\%$ for TT versus TC, $p = 0.12$, $I^2 = 46\%$ for TT versus CC, Fig. 2s). None of the other SNPs showed an association with melanoma (Figs. 3s and 4s). As the recruitment criteria for the two Leeds case–control series were different, we conducted a sensitivity analysis by removing the Leeds CCS2 data set from the meta-analyses. This made little difference to the estimated pooled OR and heterogeneity statistics.

4. Discussion

In this paper we report two UK melanoma case–control comparisons addressed primarily to understanding the role of genetic variation in *VDR* in melanoma risk. The strengths of the study are that two case–control comparisons were made from the same UK population and that the size of the first Leeds case–control study was large. A meta-analysis was also carried out on 2159 cases and 2429 controls, to provide the best evidence currently available for a relationship between the *VDR* gene and melanoma risk.

In the larger case control study (LCCS1) no relationship between *VDR* and melanoma risk was seen but in the second set there was evidence of increased risk for melanoma with the variant FokI T allele. The FokI polymorphism lies at 5′ end of the gene and results in the creation of an alternative start site, which produces a variant protein (427a.a.) that is 3 amino acids longer than the wild type protein (424a.a.). *In vitro* studies indicate that the 424a.a. wild type protein (CC) interacts more efficiently with the transcription factor TFIIB increasing trans-activation by the *VDR* compared to the T allele (427a.a.) protein.[46–48] Thus there are data to suggest that inheritance of the variant T allele is associated with reduced transcription of *VDR*-related genes, and although the second Leeds case–control series could be biased if genetic variation in *VDR* also has an effect on survival, as all cases had survived at least 3 years, these data are consistent with our observation in the published meta-analyses that the T allele is associated with melanoma risk. That the T allele has functional implications is also suggested by a recent observation that bone density in children was lower in carriers of the T allele.[49,50] The T allele of FokI has also been reported to be associated with an increased risk of colon cancer[51] and with breast cancer.[52]

In our meta-analysis the FokI T allele was associated with increased melanoma risk (OR 1.19, 95% CI 1.05–1.35), and the BsmI A allele was associated with a reduced risk (OR 0.82, 95% CI 0.71–0.94), assuming a dominant model in each case.

The meta-analysis produced evidence of a statistically significant reduced risk of melanoma in carriers of the variant BsmI A allele (Fig. 2). We did not see statistically significant evidence in support for this in either of the Leeds case–control comparisons, although the estimates from the second study were similar to those obtained in the meta-analysis. The SNP lies at the 3′end of the gene, in tight LD with the ApaI (rs7975232, C>A) SNP and a synonymous TaqI (rs731236, T>C, I352I) SNP. The 3'UTR is known to be involved in regulation of gene expression, especially via mRNA stability. Overall *in vitro* studies provide conflicting evidence for the functional effect of these polymorphisms.[13] Although many association studies have been reported, the true significance of 3′UTR variation remains unclear. For example, the *VDR* is crucial for bone health, but two large meta-analyses did not show any effect of these SNPs on fracture risk or osteoporosis.[53,54] In summary, although our meta-analysis (and those reported previously by Mocellin using fewer cases[26] and Gandini[27]) provides some evidence for a protective effect of the variant BsmI allele on melanoma risk, we did not see statistically significant evidence in support of this in our own data, and we note that the functional significance of these variants anyway remains unclear.

We report pooled evidence for increased susceptibility to melanoma associated with inheritance of the variant T allele of the FokI polymorphism of the *VDR* gene (Fig. 1). This effect is small and the significance level is modest, so that chance in the context of multiple testing is a possible explanation; the finding is consistent, however, with what is known of the effect that the variant has on *VDR* function. The study provides support for a modest role for vitamin D in melanoma susceptibility.

We also report a case–control comparison for a single serum vitamin D level at recruitment to the study. There has been only one previous study in which this was addressed but this was small and showed no evidence of a relationship between vitamin D levels in the serum and melanoma status.[55] In our study we also showed no evidence of case–control differences, but the control sample size was too small for us to exclude an effect.

We showed a relationship between vitamin D levels and hair colour. The evolution of fair skin is postulated by many to have occurred as a result of the need to manufacture vitamin D at higher latitudes, which is consistent with the observation that blonds in our study had higher levels of vitamin D than brunettes. It is of interest however that those with red hair had the lowest levels of vitamin D suggesting perhaps that more marked photosensitivity might be associated with sun avoidance so as to impact on vitamin D levels in the blood.

We have observed that BMI was related to melanoma risk, which has been reported before.[33,35,38] In one of these studies the relationship was stronger for BSA than for BMI but we did not see this in our study. As vitamin D levels are reported to be lower in obese subjects, it is not possible to allow for the possibility that the effect of obesity on melanoma risk was, at least in part, mediated by lower vitamin D levels (p-value for interaction was 0.21). It is however possible that our observation that BMI is positively associated with melanoma risk is spurious and related to a lower willingness of obese population controls to participate in the study, or to the case–control differences in socio-economic status. High BMI is also correlated with many unhealthy life-style factors, and the relationship between BMI and melanoma risk may reflect another of these factors. The relationship between obesity and melanoma risk persisted even when corrected for deprivation score ($p = 0.001$), but this adjustment is unlikely to correct for all relevant life-style factors. If BMI had an effect on melanoma risk as a result of effect on sun exposure, however, one would expect the relationship to be contrary to our findings, that is, one might postulate that the obese might be less likely to expose their skin to the sun and therefore less likely to get melanoma. The significance of the relationship between BMI and melanoma risk remains therefore unclear but it has been reported in a number of studies and therefore remains of interest.

The correlation between serum vitamin D levels and tumour Breslow thickness was marked in this study, so that patients with very thin tumours had significantly higher vitamin D levels than those with thick tumours. Berwick et al. did report better prognosis in melanoma patients who had more solar-induced skin damage on biopsy[56] and Vollmer et al. reported increased age of onset associated with solar elastosis suggestive of a protective effect of elastosis on the develop-

ment of cutaneous melanoma although there was no effect on histological prognostic markers or overall survival time.[57] Recently we have produced data supportive of an effect of serum vitamin D on survival from melanoma.[45] The data presented here suggest that higher serum vitamin D levels may be associated with reduced rate of tumour growth.

In summary, these data provide some evidence that inherited variation in *VDR* is associated with a small increased melanoma risk. We report further evidence that BMI may be related to melanoma risk, but there was no evidence for an independent relationship between lower serum vitamin D levels and risk. The study therefore supports the view that the *VDR* has a small effect on melanoma risk and shows stronger evidence that low serum vitamin D levels may be associated with tumour progression, via an effect on Breslow thickness at presentation.

Although the first Leeds case–control study described here was large, the data must be interpreted with some caution as the recruitment rate of controls was 55%. The controls furthermore had a higher socio-economic status than the cases, which is contrary to expectations, as melanoma is more common in those of higher socio-economic status in the United Kingdom (UK),[58] and therefore this likely reflects some recruitment bias. Poorer participation rates in controls are not likely to have influenced the conclusions drawn about the relationship between *VDR* and melanoma risk however and could not influence the relationship between serum vitamin D levels and tumour Breslow thickness at diagnosis. They do however make interpretation of the case–control comparisons for serum vitamin D and BMI difficult. Although we have adjusted for socio-economic status by adjusting for deprivation score, there may be residual confounding.

Conflict of interest statement

None declared.

Acknowledgements

The collection of samples in the population-ascertained sample set was funded by Cancer Research UK (Project Grant C8216/A6129) and by the NIH (R01 CA83115). Recruitment was facilitated by the UK National Cancer Research Network. The relapse study was funded by Cancer Research UK in the form of a programme grant to the Genetic Epidemiology Division (now the Section of Epidemiology and Biostatistics, Leeds Institute of Molecular Medicine, University of Leeds) of Cancer Research UK's Clinical Centre at Leeds (C588/A4994). It was also part funded by a grant from the Skin Cancer Research Fund (SCaRF), Frenchay Hospital, Bristol, BS16 1LE. The UV controls were recruited using a grant from the UK Department of Health and the Health and Safety Executive. Patrician Mack, Elaine Fitzgibbon and Kate Gamble collected data for the studies. CDHiggins, T Abramsky, Jackie Frazer, Maureen S Swanwick, Dr. John Apps (Leeds), Dr. James Bradshaw (Knaresborough), Dr. Blanchford (St. Albans) and Debbie Carson participated in the second Leeds control series.

The following recruited patients to the studies: Mr J Ausobsky, Yorkshire Clinic; Dr A Carmichael, Mr M Coady, Dr S Shehade, Mr H Siddiqui, Mr K Allison, Mr K Erdinger, Mr Ramanathan, Mr Toby Muir, James Cook University Hospital; Dr A S Highet, Mr K R Mannur, Mr M Telfer, Dr K Thomson, Mr G Miller, Mr J M Hayward, Mr J Taylor, Mr A Coatesworth, Dr A E Myatt, Dr J Schofield, Dr Callum Lyon, York District Hospital & Scarborough Hospital; Dr Alison Layton, Harrogate District Hospital; Dr Anthony Maraveyas, Dr S Walton, Dr N Alexander, Mr Alistair Platt, Mr N B Hart, Mr P M O'Hare, Mr P Stanley, Mr M Riaz, Mr Ramakrishnan, Castle Hill Hospital and Princess Royal Hospitals Hull; Dr Brunt, Mid Staffordshire General; Dr David Fyfe, Nottingham City Hospital; Dr E Marshall, Whiston Hospital; Dr D Seukeran, Friarage Hospital; Dr Bruce Pollock, Dr E D A Potts, Dr S Clark, Dr S MacDonald Hull, Mr L Le Roux Fourie, Miss O M B Austin, Mr S Southern, Mr O M Fenton, Mr S Majumder, Mr A R Phipps, Pinderfields Hospital; Dr D Cowan, Dr H Hempel, Dr J Holder, Dr M Cheesbrough, Dr H Galvin, Mr D Sutton, Huddersfield Royal Infirmary, Dr I Barbar, Calderdale Royal Hospital, Dr J A A Langtry, Dr S Natarajan, Dr Verlangi, Sunderland Royal Hospital; Dr J M Eremin, Lincoln County Hospital, Dr Lewis, Worcester Royal Infirmary; Dr M L Wood, Rotherham District General Hospital; Dr Maria Marples and Mr P W Griffiths, Weston Park Hospital, Sheffield; Dr Mark Middleton, Churchill Hospital, Oxford; Dr Marshall, Whiston Hospital; Dr Neil Cox, Cumberland Infirmary; Dr Neil Steven, Dr Peake, Queen Elizabeth Hospital Birmingham; Dr P C Lorrigan, Christie Hospital, Manchester; Dr Stewart, Walsall Manor Hospital; Dr T Watts, Lincoln County Hospital; Dr W Tucker, Alexandra Hospital; Dr Ilchyshyn, Walsgrave; Mr A R Groves, George Eliot Hospital; Mr R Debono, University Hospital North Durham; Mr Jeremy Roberts, North Staffordshire City General; Mr Murray, Wordsley Hospital & Selly Oak Hospital; Mr P Baguley, Middlesborough General Hospital; Mr Paul Davison, North Staffordshire City General; Mr R Matthews, George Eliot Hospital; Mr R B Berry, Mr M Erdmann, Mr N McLean, Mr S Rao, University Hospital North Durham; Mr S L Knight, Mr C Fenn, Dr M Marples, Mr Mark Liddington, Mr Simon Kay, Mr Howard Peach, Mr Andrew Batchelor, Dr Michelle Cronk, Dr R Sheehan-Dare, Dr Mark Goodfield, Dr Poulam Patel, Dr Victoria Goulden, Dr Alison Humphreys, Mr K Horgan, Mr Chips Browning, Dr Graeme Stables, Dr Sabine Sommer, Dr Caroline Wilson and Dr S M Wilkinson Leeds Teaching Hospitals Trust; Mr Sugden, University Hospital Hartlepool, Mr T E E Goodacre, Radcliffe Infirmary, Oxford, Dr Andrew Wright, Mr Al Ghazal, Mr S F Worrall, Mr R M Antrum, Mr Ivan Foo, Mr D Watt, Dr K London, Dr D J Barker, Prof D T Sharpe, Mr M Timmons, Bradford Royal Infirmary and Airedale Hospital, Dr G P Ford, Dr G Taylor, Dr M Shah, Dewsbury and District Hospital. These pathologists also assisted Dr A Clarke, York District Hospital. Dr A Gledhill, Harrogate, Dr A Roy, Hull. Drs Sara Edwards, Andrew Boon and Will Merchant, Leeds Teaching Hospitals Trust. Dr Srikantiah Nagara, Sunderland Hospital, Dr Paul Barrett, University Hospital North Durham, Dr David Henderson, Friarage Hospital, Dr Hugh Cochrane, Sunderland Hospital. Airedale General, Dr John J O'Dowd and Dr Phillip Batman, Bradford Royal Infirmary, Dr Patricia W Gudgeon and Dr U Raja and Dr I W Claire MacDonald, Dewsbury/Pontefract, Dr George D H Thomas,

Huddersfield Royal Infirmary, Dr Alan Padwell, Calderdale, Dr Scott Sanders, Birmingham.

We are grateful to all the clinicians who assisted in the recruitment of patients and to those patients who kindly took part.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.06.011.

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