Association of Genetic Variants of the Vitamin D Receptor (VDR) with Cutaneous Squamous Cell Carcinomas (SCC) and Basal Cell Carcinomas (BCC): A Pilot Study in a German Population

KIM KÖSTNER[1], NICOLE DENZER[1], MILENA KORENG[1], SANDRA REICHRATH[1], STEFAN GRÄBER[2], ROLF KLEIN[3], WOLFGANG TILGEN[1], THOMAS VOGT[1] and JÖRG REICHRATH[1]

[1]*Department of Dermatology and* [2]*Institut of Medical Biometry, Epidemiology and Medical Informatics, The Saarland University Hospital, Homburg, Germany;*
[3]*Seq-It GmbH and Co.KG, Kaiserslautern, Germany*

Abstract. *Background: Vitamin D receptor (VDR) polymorphisms have important implications for vitamin D signalling and are associated with various malignancies. Patients and Methods: In a German population, the frequency of several VDR polymorphisms (Apa1, Taq1, Bgl1) in basal cell carcinomas (BCCs, n=90) and cutaneous squamous cell carcinomas (SCCs, n=100) as compared to healthy controls (n=51) was analyzed. Results: Impressive variations in the frequency of some VDR genotypes were found when comparing skin of cancer patients and controls. An association of the genotype AaTtBb with BCC risk was found (BCC: 45.7%, SCC: 39.8% and controls: 38.0%). The genotype aaTTBB was exclusively found in the control group (20%), which suggested that this genotype may be protective against skin carcinogenesis. Moreover, the aaTTbb genotype was associated with skin cancer risk, being found at a much higher frequency in BCCs (21%) and SCCs (17%) as compared to controls (8.0%). Comparison of the frequencies of the VDR genotypes in sunlight-exposed vs. not sunlight-exposed skin areas revealed BB 30.1% vs. 7.1% respectively in BCCs and BB 28.1% vs. 0.0% respectively in SCCs, indicating that vitamin D signalling may be of importance for photocarcinogenesis of the skin. Associations also indicated that the Apa1 and Taq1 genotypes may be of importance for photocarcinogenesis of BCCs, but not for SCCs. Comparison of the VDR genotype frequencies by age (younger than 60 years vs. 60 years or older) revealed no evidence of age-dependent variations in patients with BCCs or SCCs. Conclusion: VDR polymorphisms are of importance for the development of BCCs and cutaneous SCCs, but further explorations of these findings and their implications are required.*

Correspondence to: Professor Dr. med. Jörg Reichrath, Department of Dermatology, The Saarland University Hospital, 66421 Homburg/Saar, Germany. Tel: +49 68411623802; Fax: +49 68411623845, e-mail: joerg.reichrath@uks.eu

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The biologically active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$, calcitrol) is responsible for a wide range of biological effects such as calcium and phosphorous regulation, apoptosis and inhibition of angiogenesis (1-3). $1\alpha,25(OH)_2D_3$ has also been shown to have significant anti-proliferative activity when administered to many cancer cells *in vitro* and regulates growth and differentiation in various cell types (4, 5). It acts by binding to a corresponding intra-nuclear vitamin D receptor (VDR), present in many target tissues (6, 7). VDR belongs to a superfamily of trans-acting transcriptional regulatory factors that includes steroid and thyroid hormone receptors as well as retinoic acid and retinoid-X receptors (8, 9). It functions as a ligand-activated transcription factor that binds to vitamin D-responsive elements (VDREs) in the promoter regions of vitamin D-responsive genes (10).

The expression and/or the function of the VDR protein can be influenced by polymorphisms in the VDR gene. Polymorphisms, defined as mutations with an allelic frequency of at least 1% in a given population, are subtle DNA sequence variations which occur often and can have biological effects. Because of their abundance in the human genome as well as their high frequencies in the human population, they have often been studied with the aim of explaining variations in the risk for common diseases (11). The VDR gene (>100 kb) encompasses two promoter regions, eight protein-coding exons namely 2-9 and six untranslated exons (1a-1f) (12). More than sixty VDR polymorphisms have been discovered which are located in the promoter, in

Table I. *Primer sequences for the analysis of VDR polymorphisms (ApaI, TaqI and BglI).*

	Polymorphism	Primer no.		Sequence
DNA isolated from blood	*Apa*I + *Taq*I	Primer 1	Forward	5'GTAAAACGACGGCCAGTGTGGTATCACCGGTCAAGCAGTC3''
			Reverse	5'CACTCAGGCTGGAAGGAGAGG3'
	*Bgl*I	Primer 2	Forward	5'GTAAAACGACGGCCAGTGCCTATCCACCCAGCCCATTCT3'
			Reverse	5'TCTCTGTCCCTGAGGAATGGA3'
DNA isolated from tissue	*Apa*I	Primer 3	Forward	5' CAGGACGATCTGTGGGCAC3'
			Reverse	5'GTAAAACGACGGCCAGTGCAGGACGATCTGTGGGCAC 3'
	*Taq*I	Primer 4	Forward	5'TGAGAGCTCCTGTGCCTTCTT3'
			Reverse	5'TGTTGGACAGGCGGTCC5'
	*Bgl*I	Primer 5	Forward	5'GGCCTTGCCCAGAGATGCC3'
			Reverse	5'TCTCTGTCCCTGAGGAATGGA3'

and around exons 2-9 and in the 3`untranslated (UTR) region (13, 14). Most of the VDR polymorphisms are restriction fragment length polymorphisms (RFLP) with an unknown functional effect. In some cases, it has been indicated that they may be linked to truly functional polymorphisms elsewhere in the VDR gene (or in a nearby gene) (13).

It is now accepted that polymorphisms along the 105 kilobyte VDR gene have important implications for vitamin D signalling and are associated with various malignancies, including cancer of the breast, colon, prostate and skin (15, 16). However, little is known about the role of the vitamin D endocrine system for carcinogenesis of BCCs and SCCs.

Ultraviolet radiation is known to have strong carcinogenic effects on skin tissue which makes it a strong risk factor for non-melanoma skin carcinomas such as BCC and SCC (17, 18). Although it is obvious that the relationship between UV radiation and skin cancer is more complex than for other types of cancer, there is evidence of a protective effect of vitamin D. Sunlight causes DNA damage, but also induces the production of vitamin D whose metabolite $1,25(OH)_2D_3$ has anti-proliferation and pro-differentiation effects in both melanocytes and cutaneous melanoma (CM) cells, mediated through the VDR. Most studies investigating the association of polymorphisms and increased risk of skin cancer have been performed for malignant melanoma. The restriction fragment length polymorphisms (RFLPs) *Taq*I (19), *Fok*I (19, 20), and *Bsm*I (16, 21, 22) have been reported to affect the risk of malignant melanoma. Some associations of the *Bsm*I polymorphism with SCC (22) and of BCC (23) have also been reported.

In the present study, a gene sequencing approach was used to analyse the frequency of three previously described RFLPs (13-23) in the VDR gene (*Apa*I: rs7975232, located in the last intron of the VDR gene; *Taq*I: rs731236, located in exon 9; *Bgl*I: rs739837, lying 303 bp downstream of the stop codon) in BCC and SCC as compared to healthy controls. Moreover, other factors such as tumor localisation, gender and age were examined to test the hypothesis that VDR polymorphisms were associated with skin cancer risk, possibly by interacting with these factors.

Materials and Methods

Subjects. Formalin-fixed paraffin-embedded tissues of BCCs (n=90) and SCCs (n=100) were used. The tissues were obtained from the tissue bank of the Dermatology Department, Saarland University Hospital. The diagnoses of the cases were confirmed by certified histopathologists in the Dermatology Department. The control group consisted of 51 healthy volunteers (age range 20-40 years). This study was approved by the Ethical committee of the *Ärztekammer des Saarlandes*.

DNA isolation from BCCs and SCCs. The required thin (10×10 μm) tissue sections on slides were dried at 37˚C overnight. After soaking the tissue sections in xylene, deparaffination was carried out with a graded ethanol series for 3-5 min each (100% ethanol for dehydration, 80% ethanol, 60% ethanol and 40% ethanol). To differentiate healthy from tumor tissue, the slide was stained with hematoxylin. The tumor tissue was isolated by microdissection and the DNA was extracted with a DNA isolation kit (High Pure PCR Template Preparation Kit, Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

DNA isolation from blood of healthy volunteers (control group). Genomic DNA of the controls was extracted from 200 μl EDTA blood with the DNA isolation kit from Roche Diagnostics (High Pure PCR Template Preparation Kit) according to the manufacturer's instructions.

To obtain higher DNA concentrations, with some blood samples, lymphocyte separation was first performed according to the manufacturer's instructions (PAA Laboratories, Cölbe, Germany). Briefly, 3 ml diluted blood samples were carefully layered onto 3 ml Ficoll 400 and centrifuged at 1,200 × *g* for 20 min at RT, and lymphocytes from the interphase were washed twice in PBS. After that the DNA was isolated as above.

Genotyping of VDR polymorphisms (SNPs). The forward and reverse primers used to genotype the three RFLPs in the controls and subjects are shown in Table I. The *Apa*I and *Taq*I polymorphisms of the control group (DNA isolated from peripheral venous blood samples) were amplified within the same PCR using

Table IIA. *VDR genotypes in basal cell carcinomas (BCC).*

Genotype	Cases		Controls		*P*-value
	No.	%	No.	%	
*Apa*I					
aa	18	22.0	14	27.5	0.467
Aa	46	56.1	23	45.1	
AA	18	22.0	14	27.5	
	82	100.0	51	100.0	
*Apa*I homozygotes *vs*. heterozygotes					
AA/aa	36	43.9	28	54.9	0.284
Aa	46	56.1	23	45.1	
	82	100.0	51	100.0	
*Taq*I					
tt	11	12.6	7	14.0	0.461
Tt	51	58.6	24	48.0	
TT	25	28.7	19	38.0	
	87	100.0	50	100.0	
*Taq*I homozygotes *vs*. heterozygotes					
TT/tt	36	41.4	26	52.0	0.285
Tt	51	58.6	24	48.0	
	87	100.0	50	100.0	
*Bgl*I					
bb	17	19.3	14	27.5	0.383
Bb	48	54.5	22	43.1	
BB	23	26.1	15	29.4	
	88	100.0	51	100.0	
*Bgl*I homozygotes *vs*. heterozygotes					
BB/bb	40	45.5	29	56.9	0.221
Bb	48	54.5	22	43.1	
	88	100.0	51	100.0	

Table IIB. *VDR genotypes in cutaneous squamous cell carcinomas (SCC).*

Genotype	Cases		Controls		*P*-value
	No.	%	No.	%	
*Apa*I					
aa	19	21.6	14	27.5	0.702
Aa	45	51.1	23	45.1	
AA	24	27.3	14	27.5	
	88	100.0	51	100.0	
*Apa*I homozygotes *vs*. heterozygotes					
AA/aa	43	48.9	28	54.9	0.598
Aa	45	51.1	23	45.1	
	88	100.0	51	100.0	
*Taq*I					
tt	19	19.0	7	14.0	0.607
Tt	50	50.0	24	48.0	
TT	31	31.0	19	38.0	
	100	100.0	50	100.0	
*Taq*I homozygotes *vs*. heterozygotes					
TT/tt	50	50.0	26	52.0	0.864
Tt	50	50.0	24	48.0	
	100	100.0	50	100.0	
*Bgl*I					
bb	24	24.0	14	27.5	0.727
Bb	50	50.0	22	43.1	
BB	26	26.0	15	29.4	
	100	100.0	51	100.0	
*Bgl*I homozygotes *vs*. heterozygotes					
BB/bb	50	50.0	29	56.9	0.492
Bb	50	50.0	22	43.1	
	100	100.0	51	100.0	

Primer 1 (Table I). PCR and sequencing were performed by *Seq-It GmbH* and *Co.KG*, Kaiserslautern, Germany.

PCR and gel electrophoresis. The PCR amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Darmstadt, Germany) with Thermo-Fast-PCR-plates (Abgene, Epsom, UK). The total 15.2 μl of the PCR reaction consisted of 1.5 μl 10 × PCR buffer, 0.54 μl $MgCl_2$ (50 mM), 0.2 μl dNTPs (10 mM), 0.15 μl BSA (20 ng/μl), 0.12 μl Platinum *Taq* polymerase, Primermix and 50 ng isolated DNA. PCR was performed with an initial denaturation at 94˚C for 2 min, followed by 33 cycles of denaturation at 95˚C for 10 s, annealing at 60˚C for 30 s and extension at 72˚C for 30 s. The PCR products were size-separated on a 2% agarose gel at 100 V for 20 min.

Sequencing. To sequence the region around the polymorphisms *Apa*I, *Taq*I and *Bgl*I, the amplified PCR products were purified. Direct sequence analysis of the PCR products was performed by cycle sequencing using an ABI PRISM bigDye kit from Applied Biosystems. The sequencing reaction (7 μl) included 0.8 μl BigDye, 1.6 μl 5 × buffer (400 mM Tris-Cl pH 9.0, 10 mM $MgCl_2$), 6 μM primer, respectively and 2 μl of PCR product. The sequencing reaction was performed in a Gene Amp PCR System 9700 (Applied Biosystems) with the following 31 cycles: 96˚C for 7 s, 50˚C for 10 s and 60˚C for 2 min. Consistent with the literature, genotypes for the three RFLP polymorphisms are reported using standard nomenclature for RFLP assays (using lower and upper case letters to indicate the presence or absence of a restriction site, respectively.) The *Apa*I *G* and *T* alleles are indicated by *a* and *A*, respectively; the *Taq*I *T* and *C* alleles by *T* and *t* respectively, and the *Bgl*I *C* and *A* by *b* and *B*, respectively.

Statistical analysis. The frequencies of the polymorphisms between the different groups were compared with the chi-square or the Fisher's exact test. The significance cut-off value was $p \leq 0.05$.

Results

VDR genotype and allele frequencies. The genotype and allele frequencies for the *Apa*I, *Taq*I and *Bgl*I polymorphisms in the BCC patients and controls can be seen in Table IIA. In general, the most frequent genotype both in the BCC patients and the controls was the heterozygous genotype with

Aa (56.1% in patients, 45.1% in controls), Tt (58.6% in patients, 48.0% in controls) and *Bb* (54.5% in patients, 43.1% in controls) alleles. There was trend towards a higher distribution of homozygous genotypes for all three polymorphisms in the controls than the BCCs (54.9% *vs*. 43.9% for *Apa*I, 52.0 *vs*. 41.4% for *Taq*I and 56.9% *vs*. 45.5% for *Bgl*I), but these differences were statistically not significant (Table IIA).

Table IIB shows the genotype frequencies of the *Apa*I, *Taq*I and *Bgl*I polymorphisms in the SCC patients and controls. No significant differences in the frequency of individual genotypes comparing SCC patients and controls were found.

Analyzing the combined *Apa*I/*Taq*I/*Bgl*I genotype, an association of the genotype *AaTtBb* with BCC risk was found (BCC: 45.7%, SCC: 39.8% and controls: 38.0%) (Table III). There was no difference in the distribution of the *AAttBB* genotype between cases and controls (BCC: 12.3%, SCC: 13.6% and controls 14.0%). The genotype *aaTTBB* was exclusively found in the control group (20%). Moreover, the *aaTTbb* genotype was associated with skin cancer risk, being found at a much higher frequency in the BCCs (21%) and SCCs (17%) as compared to the controls (8.0%) (Table III).

Association of VDR genotypes with localization of BCCs or SCCs. The frequencies of the VDR genotypes in sunlight-exposed *vs*. not sunlight-exposed skin areas were compared (Tables IVA and B). In the BCCs, the BB genotype was 30.1% *vs*. 7.1% and in the SCCs, BB was 28.1% *vs*. 0.0% in sunlight exposed *vs*. not sunlight-exposed skin areas, respectively. However, the associations of this VDR polymorphism with skin cancer risk were statistically not significant (Tables IVA and B). Associations indicated that the *Apa*I and *Taq*I genotypes may be of importance for photocarcinogenesis of BCC, but not for SCC (Tables IVA and B).

VDR genotypes and age of BCC and SCC patients. Comparison of the frequencies of the VDR genotypes in patients younger than 60 years *vs*. 60 years or older revealed no evidence of age-dependent variations in patients with BCCs or SCCs (Tables VA and B).

Discussion

Recently, it has been shown that vitamin D_3 inhibits hedgehog signaling and proliferation in murine BCCs (24). This observation is of particular importance, for constitutive Hedgehog (HH) signaling underlies several human tumors, including BCC (24). Interestingly, it has been reported that topical vitamin D_3 treatment of existing murine BCC tumors significantly decreased Gli1 and Ki67 staining, indicating that topical vitamin D_3 acting *via* its HH inhibiting effect may hold promise as an effective anti-BCC agent (24).

Table III. *The combined ApaI/TaqI/BglI genotypes in BCCs, SCCs and controls*.

Genotype	BCC		SCC		Controls		Total	
	No.	%	No.	%	No.	%	No.	%
aaTTbb	17	21.0	15	17.0	4	8.0	36	16.4
aaTTBB	0	0.0	3	3.4	10	20.0	13	5.9
aaTtBb	0	0.0	1	1.1	0	0.0	1	0.5
AaTtbb	0	0.0	1	1.1	1	2.0	2	0.9
AaTtBb	37	45.7	35	39.8	19	38.0	91	41.6
AaTtBB	1	1.2	2	2.3	0	0.0	3	1.4
AattBB	1	1.2	0	0.0	0	0.0	1	0.5
AaTTBb	7	8.6	8	9.1	2	4.0	17	7.8
AAttbb	0	0.0	5	5.7	0	0.0	5	2.3
AAttBB	10	12.3	12	13.6	7	14.0	29	13.2
AATtBB	8	9.9	5	5.7	5	10.0	18	8.2
AATTBB	0	0.0	1	1.1	2	4.0	3	1.4

Notably, associations of some of the VDR genotypes with skin cancer risk were found. However, some of these associations were statistically not significant, most likely due to the relatively low case numbers. In general, associations of the VDR genotypes with skin cancer risk were stronger for the BCCs compared to the SCCs, indicating that VDR-mediated signalling has a greater relevance for the pathogenesis of BCCs than SCCs. A higher frequency of homozygous genotypes for all three polymorphisms was found in the controls than the BCCs and an association of the genotype *AaTtBb* with BCC risk was found. Interestingly, the genotype *aaTTBB* was exclusively found in the control group (20%), which suggested that this genotype may be protective against skin carcinogenesis. Moreover, a higher frequency of the *aaTTbb* genotype was found in the BCCs and SCCs as compared to the controls. Thus this study indicated that VDR polymorphisms are associated with skin cancer risk, supporting the increasing body of evidence demonstrating an important role of the cutaneous vitamin D endocrine system in the prevention of photocarcinogenesis.

The TT allele of the *Taq*I polymorphism has been reported to be associated with lower circulating levels of active vitamin D_3, although the *Taq*I polymorphism leads to a silent codon change (from ATT to ATC, which both result in an isoleucine at codon 352) (25, 26). The *Taq*I allele *t* has been reported to be significantly less frequent among melanoma cases than among controls (25, 27), suggesting that *t* might protect carriers against melanoma or *T* might put them at risk.

The ApaI RFLP is located in intron 8 at the 3' end of the VDR gene (25, 28). It was reported that the *Apa*I, *Bsm*I, and *Taq*I polymorphisms were in strong linkage-disequilibrium (LD) (25, 29). In melanoma patients, there was no evidence of any association between any haplotype and melanoma status (25, 29). One study assessed the possible implications

Table IVA. *VDR genotypes and localization of BCC.*

Genotype	Sun exposure		No sun exposure		Total		*P*-value
	No.	%	No.	%	No.	%	
*Apa*I							
AA	17	25.4	1	7.1	18	22.2	0.214
Aa	37	55.2	8	57.1	45	55.6	
aa	13	19.4	5	35.7	18	22.2	
	67	100.0	14	100.0	81	100.0	
*Taq*I							
TT	18	25.0	6	42.9	24	27.9	0.443
Tt	44	61.1	7	50.0	51	59.3	
tt	10	13.9	1	7.1	11	12.8	
	72	100.0	14	100.0	86	100.0	
*Bgl*I							
BB	22	30.1	1	7.1	23	26.4	0.106
Bb	39	53.4	8	57.1	47	54.0	
bb	12	16.4	5	35.7	17	19.5	
	73	100.0	14	100.0	87	100.0	

Table IVB. *VDR genotypes and localization of SCC.*

Genotype	Sun exposure		No sun exposure		Total		*P*-value
	No.	%	No.	%	No.	%	
*Apa*I							
AA	23	29.1	1	20.0	24	28.6	1.000
Aa	39	49.4	3	60.0	42	50.0	
aa	17	21.5	1	20.0	18	21.4	
	79	100.0	5	100.0	84	100.0	
*Taq*I							
TT	28	31.5	2	33.3	30	31.6	1.000
Tt	44	49.4	3	50.0	47	49.5	
tt	17	19.1	1	16.7	18	18.9	
	89	100.0	6	100.0	95	100.0	
*Bgl*I							
BB	25	28.1	0	0.0	25	26.3	0.202
Bb	43	48.3	3	50.0	46	48.4	
bb	21	23.6	3	50.0	24	25.3	
	89	100.0	6	100.0	95	100.0	

Table VA. *VDR genotypes and age in BCC patients.*

Genotype	<60 years		≥ 60 years		Total		*P*-value
	No.	%	No.	%	No.	%	
*Apa*I							
AA	3	30.0	14	20.0	17	21.3	0.815
Aa	5	50.0	40	57.1	45	56.3	
aa	2	20.0	16	22.9	18	22.5	
	10	100.0	70	100.0	80	100.0	
*Taq*I							
TT	3	30.0	21	28.0	24	28.2	1.000
Tt	6	60.0	45	60.0	51	60.0	
tt	1	10.0	9	12.0	10	11.8	
	10	100.0	75	100.0	85	100.0	
*Bgl*I							
BB	3	30.0	19	25.0	22	25.6	1.000
Bb	5	50.0	42	55.3	47	54.7	
bb	2	20.0	15	19.7	17	19.8	
	10	100.0	76	100.0	86	100.0	

Table VB. *VDR genotypes and age in SCC patients.*

Genotype	<60 years		≥ 60 years		Total		*P*-value
	No.	%	No.	%	No.	%	
*Apa*I							
AA	1	20.0	23	27.7	24	27.3	0.702
Aa	2	40.0	43	51.8	45	51.1	
aa	2	40.0	17	20.5	19	21.6	
	5	100.0	83	100.0	88	100.0	
*Taq*I							
TT	2	40.0	29	30.9	31	31.3	1.000
Tt	2	40.0	48	51.1	50	50.5	
tt	1	20.0	17	18.1	18	18.2	
	5	100.0	94	100.0	99	100.0	
*Bgl*I							
BB	1	20.0	24	25.5	25	25.3	0.712
Bb	2	40.0	48	51.1	50	50.5	
bb	2	40.0	22	23.4	24	24.2	
	5	100.0	94	100.0	99	100.0	

of the *Apa*I polymorphism for solar keratosis (SK) prevalence (25, 30). SKs are established biomarkers for BCC and SCC. It is nowadays accepted that most SCCs derive from SKs and that SKs represent SCCs *in situ*. In individuals with fair skin, the prevalence of SK was higher in the homozygote groups with *AA* or *aa* than in the heterozygote group with *Aa* (eightfold *vs*. fivefold) compared with heterozygote groups with medium or olive skin (25, 30). It has been speculated that the heterozygote genotype Aa may protect individuals against being affected by SK, in conjunction with skin colour or tanning ability (25, 30).

Data on the *Bgl*I polymorphism and skin cancer or cancer in general are still very limited. Barroso *et al*. observed no statistically association with MM and the *Bgl*I polymorphism (31). Only a marginally significant association with fair skin colour (p=0.048) and with Fitzpatrick's phototype I/II (p=0.070) was seen (31).

The relevance of individual VDR polymorphisms for vitamin D signalling has been demonstrated (25). A strong LD has also been noticed at the 3' end of the VDR gene for the *Bsm*I, *Apa*I and *Taq*I RFLP's (25, 29, 32). The most frequent haplotypes were *baT* (48%) and *Bat* (40%) (33). *In*

vitro functional studies have demonstrated that the *baT* haplotype inserted into transfection constructs resulted in lower reporter gene activity compared with *Bat* and was associated with low VDR messenger RNA levels (34, 35). It has been speculated that this reduction in vitamin D-mediated transcriptional activity may lead to an increased melanoma risk (25). However, it has to be noted that individual VDR polymorphisms cannot be regarded as independent prognostic factors (25). Vitamin D signaling depends on a broad variety of additional factors that include 25-hydroxyvitamin D serum levels and vitamin D metabolism. Data indicate that vitamin D serum levels are associated with risk for and prognosis of melanoma (36, 37).

In the present study, we have also analyzed whether VDR polymorphisms are associated with skin tumor risk in different age groups and in chronically sunlight-exposed *vs*. not chronically sunlight-exposed skin areas. A trend indicated that *Apa*I and *Taq*I genotypes may be of importance for photocarcinogenesis of BCC, but not for SCC. The VDR genotypes did not play a major role in determing the time of onset of BCC or SCC.

In summary, this pilot study in a German population indicates that VDR polymorphisms may be of importance for the development of BCC and cutaneous SCC, but further exploration of these findings and their implications is required.

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