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Impaired Vitamin D Activation and Association with *CYP24A1* Haplotypes in Differentiated Thyroid Carcinoma

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Background: Common polymorphisms of the vitamin D receptor gene have been reported to affect the risk of breast, colon, prostate, and differentiated thyroid cancer (DTC), but polymorphisms within the genes of vitamin D metabolizing enzymes have not been studied in DTC. The aim of the present study was to investigate the genes for vitamin D enzymes in patients with DTC and healthy controls (HC) as well as the vitamin D (25-hydroxyvitamin D_3, and 1,25-hydroxyvitamin) status.

Methods: German patients ($n = 253$) with DTC (papillary thyroid carcinoma [PTC] and follicular thyroid carcinoma [FTC]) and HC ($n = 302$) were genotyped for polymorphisms within the vitamin D metabolizing enzymes such as 25-hydroxylase (*CYP2R1*[rs12794714, rs10741657]), 25-hydroxyvitamin D-1α-hydroxylase (*CYP27B1*[rs10877012, rs4646536]), and 25-hydroxyvitamin D 24-hydrolase (*CYP24A1*[rs927650, rs2248137, rs2296241]). Furthermore, the 25-hydroxyvitamin D_3 [$25(OH)D_3$] and 1,25-hydroxyvitamin [$1,25(OH)_2D_3$] plasma levels were measured by a radioimmunoassay.

Results: There was no difference in the genotypes; however, the *CYP24A1* haplotype analysis showed that *rs2248137C/rs2296241A* (13.1% vs. 19.1%; corrected p [pc] = 0.04) was less frequent in the PTC, whereas the haplotypes *rs2248137C/rs2296241G* (56.0% vs. 41.9%; $pc = 0.03$), *rs927650C/rs2296241G* (22.5% vs. 8.4%; $pc = 1.6 \times 10^{-3}$), and *rs927650C/rs2248137C/rs2296241G* (21.1% vs. 7.3%; $pc = 1.5 \times 10^{-3}$) were more frequent in the FTC compared with HC. Furthermore, if patients and controls were grouped according to four $25(OH)D_3$ categories (severely deficient, deficient, insufficient, and sufficient), then the patients with both DTC subtypes had significantly lower levels of circulating $1,25(OH)_2D_3$, especially in the group with a deficient $25(OH)D_3$ status compared with the controls. Although the polymorphisms showed no differences stratified for the four $25(OH)D_3$ categories, the activation status by $1,25(OH)_2D_3$ differed significantly depending on the genotypes of the investigated *CYP24A1* polymorphisms.

Conclusions: A higher risk for DTC is conferred by haplotypes within the *CYP24A1* gene, low circulating $25(OH)D_3$ levels (deficiency), and a reduced conversion to $1,25(OH)_2D_3$. These results confirm and extend previous observations and also support a role of the vitamin D system in the pathogenesis of DTC. How deficient $25(OH)D_3$ levels in combination with certain *CYP24A1* haplotypes affect vitamin D activation is the subject of future studies.

Introduction

Thyroid cancer is the most common malignancy of the endocrine system, representing ~1% of all neoplasias (1). Among them, differentiated thyroid carcinoma (DTC) includes papillary (85% of cases) and follicular (10% of cases) subtypes as the most frequent (2). Thyroid carcinogenesis is a multistep process involving a multifactorial interplay between genetic and environmental factors. Environmental exposure to lower levels of ultraviolet (UV) radiation, which is essential for the synthesis of vitamin D, may be a risk factor for higher cancer incidence of many types (3). Furthermore, we earlier reported an altered vitamin D system in DTC, including lower circulating 1,25-hydroxyvitamin D as well as an association with vitamin D receptor (VDR) polymorphisms (4). This led us to investigate whether vitamin D status is useful to

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link genetic factors and the environment in the pathogenesis of thyroid malignancies. Specific enzymes regulate the synthesis and degradation of 25-hydroxyvitamin D_3 [calcidiol; $25(OH)D_3$] and 1,25 hydroxyvitamin D_3 [calcitriol; $1,25(OH)_2D_3$], whose genetic polymorphisms may modify the risk for DTC by changing the bioavailability of $25(OH)D_3$ and $1,25(OH)_2D_3$.

The photochemical synthesis of vitamin D_3 (cholecalciferol) occurs cutaneously, where provitamin D_3 (7-dehydrocholesterol) is converted to previtamin D_3 in response to UV sunlight exposure. Vitamin D_3 obtained from the isomerization of previtamin D_3 is hydroxylated in the liver through the cytochrome P450 enzyme, 25-hydroxylase (CYP2R1) to form $25(OH)D_3$, the major circulating form of vitamin D (5). In the kidney, $25(OH)D_3$ is activated by 1α-hydroxylase (CYP27B1) to the metabolite $1,25(OH)_2D_3$ (6). $1,25(OH)_2D_3$ binds to the nuclear VDR in target organs, forming heterodimers with the retinoid X receptor and recruiting other transcriptional cofactors that regulate target gene transcription, including those involved in cell proliferation, differentiation, and apoptosis (7–9). The final step in the vitamin D metabolism pathway is the degradation of $25(OH)D_3$ and $1,25(OH)_2D_3$ to $24,25(OH)D_3$ and $24,25(OH)_2D_3$, respectively, which occurs through 24-hydroxylation by 25-hydroxyvitamin D 24-hydrolase (CYP24A1) (10). The aim of this study was to investigate the polymorphisms within the vitamin D metabolizing genes *CYP2R1* (rs12794714, rs10741657), *CYP27B1* (rs10877012, rs4646536), and *CYP24A1* (rs927650, rs2248137, rs2296241) and their influence on vitamin D levels [$25(OH)D_3$ and $1,25(OH)_2D_3$] in DTC.

Methods

Subjects

In total, 253 patients (167 women and 86 men) with a pathologically confirmed diagnosis of DTC (205 papillary: 137 women and 68 men and 48 follicular: 30 women and 18 men) and known tumor-node-metastasis stage were recruited from the Departments of Medicine 1 and of Nuclear Medicine in the University Hospital as well as from the Department of Surgery, Bürgerhospital, Frankfurt am Main, Germany. Healthy controls (HC; $n = 302$, 138 women and 164 men) were volunteer blood donors from the staff or medical students without a family history of thyroid carcinoma. All participants were of German origin and inhabitants of the area surrounding Frankfurt am Main, Germany. The median age of the patients with DTC and HC was 55 and 38 years, respectively. The study protocol was approved by the ethics committee of the University Hospital Frankfurt am Main, and informed consent was obtained from all participants.

Genes and polymorphisms

In total, seven polymorphisms were investigated: two for 11p15/*CYP2R1* (length 15.5 kb, 5 exons), two for 12q13/*CYP27B1* (length 4.8 kb, 9 exons), and three for 20q13/*CYP24A1* (length 20.5 kb, 12 exons) were selected. Polymorphism positions are given according to the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov): The rs12794714 (T/C, Ser→Ser) is a synonymous polymorphism in exon 2 of CYP2R1, whereas the polymorphism rs10741657 (G/C) is mapped to a 2-kb mRNA transcript. The rs10877012 polymorphism within the *CYP27B1* (−1260 C/A) is located in the promoter region, exactly in the AP-2 transcription-factor binding motif and the rs4646536 polymorphism (+2838 C/T) in intron 6. While the rs927650 (C/T) and rs2248137 (C/G) are intronic polymorphisms, the rs2296241 (A/G) is synonymous in exon 4 of the *CYP24A1* gene.

Genotyping

Genomic DNA was extracted from whole blood by the salting-out procedure (11) and was used for restriction fragment length polymorphism and real-time polymerase chain reaction (PCR) methods. For the *CYP2R1* (rs12794714, rs10741657), the DNA was amplified with the primer pairs and PCR conditions as previously described (12). The *CYP24A1* rs927650 polymorphism was examined using the primers 5′-TGGTTGCATAACACA AACCTA-3′ and 5′-CTGAAAGCCAGTAACAATGGT-3′. The annealing temperature was 59°C. The amplified DNA for rs12794714 and rs10741657 polymorphisms (288-bp and 303-bp, respectively) were digested with enzymes (*Mnl*I and *Fok*I, respectively; New England Biolabs®) according to the manufacturer's instructions. The resulting 311-bp PCR product for rs927650 was digested with *BgL*II and revealed two fragments of 210-bp and 101-bp if the allele T was present. The digestion products were separated on 3% agarose gel visualized by ethidiumbromide staining. The polymorphisms within the *CYP24A1* gene (rs2248137/C_1915659; rs2296241/C_19156 56_30) and *CYP27B1* gene (rs4646536/C_25623453; rs10877012/ assay-by-design) were analyzed using Taqman assays in an ABI 7300 PCR system under the conditions recommended by the manufacturer (Applied Biosystems). Finally, the *CYP27B1* rs10877012 promoter polymorphism was studied using the following primer and probe sequences: forward, GGG-AGTAAGGAGCAGAGAGGTAAA; reverse, AACAGAGA-GAGGGCCTGTCT; FAM-labelled probe for C allele, TGTGGGAGATTCTTTTA; VIC-labeled probe for A allele, CTGTGGGAGATTATTTTA (13). In order to confirm the accuracy of the methods, random samples of the studied polymorphism were genotyped twice with a concordance of 98%.

Measurement of $25(OH)D_3$ and $1,25(OH)_2D_3$

$25(OH)D_3$ and $1,25(OH)_2D_3$ were measured by a radioimmunoassay (DiaSorin and IDS, respectively) in the plasma of patients with DTC and HC. For the determination of both metabolites, fresh ethylenediaminetetraacetic acid blood samples were immediately centrifuged at 600 *g* for 10 minutes, and separated plasma was stored at −20°C. The blood samples of the patients and controls were obtained in the period from October 2006 to December 2008 and from April 2007 to September 2009, respectively. We defined German seasons as winter (November–March) and summer (April–October). Patients with papillary thyroid cancer (PTC) and follicular thyroid carcinoma (FTC) as well as control subjects were sampled more frequently in the summer (67%, 71%, and 83%, respectively). Circulating levels of $25(OH)D_3$ were defined as being severely deficient (< 10 ng/mL or < 25 nM), deficient (10–20 ng/mL or 25–50 nM), insufficient (20–30 ng/mL or 50–75 nM), and sufficient (> 30 ng/mL or > 75 nM) (14). The plasma concentration of $1,25(OH)_2D_3$ was considered normal in the range of 19.9–67 pg/mL according to the manufacturer's instructions.

Clinical parameters

The parathyroid hormone levels were not available for the patients with DTC. For the evaluation of the renal function,

Table 1. Distribution of *CYP2R1* (rs12794714, rs10741657), *CYP27B1* (rs10877012, rs4646536), and *CYP24A1* (rs927650, rs2248137, and rs2296241) Gene Polymorphisms in Patients with Papillary Thyroid Cancer, Follicular Thyroid Cancer, and Healthy Controls

	HC, n = *302*		*PTC*, n = *205*				*FTC*, n = *48*			
Genotypes	n	%	n	%	p	pc	n	%	p	pc
CYP2R1 rs12794714										
GG	94	31.1	65	31.7			13	27.1		
AG	144	47.7	104	50.7	0.59	1.77	26	54.2	0.70	2.1
AA	64	21.2	36	17.6			9	18.7		
CYP2R1 rs10741657										
GG	119	39.4	75	36.6			21	43.8		
GA	139	46.0	91	44.4	0.40	1.20	19	39.5	0.70	2.1
AA	44	14.6	39	19.0			8	16.7		
CYP27B1 rs10877012										
CC	134	44.4	99	48.3			18	37.5		
AC	126	41.7	84	41.0	0.50	1.50	24	50.0	0.56	1.68
AA	42	13.9	22	10.7			6	12.5		
CYP27B1 rs4646536										
TT	138	45.7	96	46.8			18	37.5		
TC	122	40.4	87	42.5	0.57	1.71	24	50.0	0.45	1.35
CC	42	13.9	22	10.7			6	12.5		
CYP24A1 rs927650										
TT	79	26.1	44	21.5			9	18.7		
CT	150	49.7	99	48.3	0.24	0.72	27	56.3	0.53	1.59
CC	73	24.2	62	30.2			12	25.0		
CYP24A1 rs2248137										
CC	116	38.4	73	35.6			24	50.0		
CG	136	45.0	89	43.4	0.44	1.32	18	37.5	0.31	0.93
GG	50	16.6	43	21.0			6	12.5		
CYP24A1 rs2296241										
AA	89	29.5	57	27.8			9	18.8		
AG	151	50.0	101	49.3	0.79	2.37	22	45.8	**0.05**	0.15
GG	62	20.5	47	22.9			17	35.4		

p-Value = 0.05 is boldface.
HC, healthy controls; PTC, papillary thyroid cancer; FTC, follicular thyroid carcinoma; *pc*, corrected *p*.

creatinine values (patients: $n = 136$) were documented and found to be normal (median = 0.9 mg/dL; normal range 0.66–1.09 mg/dL). The calcium values of 172 patients were provided. The calcium concentration was in a normal range (2.1–2.55 mM) in most of the patients with DTC (90%). Hypocalcemia (< 2.1 mM) was found in 15 patients (8.7%), and hypercalcemia (> 2.55 mM) was found in 2 patients (1.3%). The determination of creatinine and calcium was performed using the Cobas® analyzer (Roche Diagnostics). The reference range was obtained from the manufacturer of the measuring system.

Statistical analysis

Deviation from the Hardy–Weinberg equilibrium, differences in genotype, and allele distributions between groups were evaluated by χ^2-test (BiAS software, package 9.08; Epsilon). The haplotype frequencies for the *CYP2R1*, *CYP27B1*, and *CYP24A1* polymorphisms as well as the linkage disequilibrium (LD) between markers were estimated using Haploview software version 3.11 (www.broad.mit.edu/mpg/haploview). The odds ratio (OR) and its 95% confidence interval (CI) were estimated by unconditional logistic regression as a measure of the associations between haplotypes and thyroid cancer risk. The *p*-values were corrected by multiplication with the number of genotypes, alleles, or haplotypes tested (Bonferroni correction). Power calculation for each group was performed assuming an allele frequency of 41.7% (derived from controls used in this study) and a type 1 error rate of 5%. On the basis of these assumptions, we estimate that we have a power > 80% to detect an allelic OR > 2.40 or < 0.38 (FTC) and > 1.66 or < 0.59 (PTC) for disease susceptibility in the case/control data set by using the software Power and Sample Size Calculations 2.1.30 (http://biostat.mc.vanderbilt.edu/PowerSampleSize). Since the $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations were not usually distributed according to the Kolmogoroff–Smirnoff test; nonparametric tests were used for the comparisons between the groups (Wilcoxon–Mann–Whitney and Kruskal–Wallis test; BiAS software). Furthermore, vitamin D levels were correlated with genotypes. These comparisons were performed using the Kruskal–Wallis test. In case the *p*-value of the Kruskal–Wallis test was < 0.05, then a multiple Conover–Iman comparison between the groups was performed, and the *p*-values were corrected (Bonferroni correction). A *p*-value of < 0.05 was considered significant.

Results

Genotype analysis

All samples were in the Hardy–Weinberg Equilibrium ($p > 0.05$) for each polymorphism. No significant differences were observed in the genotype (Table 1) and allele (data not shown) frequencies between patients with PTC, FTC, and HC

Table 2. Comparison of *CYP24A1* Haplotypes Derived from rs927650, rs2248137, and rs2296241 in Patients with Papillary Thyroid Cancer, Follicular Thyroid Cancer, and Healthy Controls

	HC, n = *302*	*PTC*, n = *205*					*FTC*, n = *48*				
Haplotypes	*F (%)*	*F (%)*	*OR*	*[95% CI]*	p	pc	*F (%)*	*OR*	*[95% CI]*	p	pc
rs2248137/rs2296241											
CA	19.1	13.1	0.64	[0.45–0.91]	**0.01**	**0.04**	12.7	0.62	[0.33–1.17]	0.14	0.42
CG	41.9	44.2	1.00	[0.86–1.42]	0.51	0.54	56.0	1.77	[1.14–2.73]	**0.01**	**0.03**
GA	35.4	39.4	1.18	[0.91–1.53]	0.23	0.71	28.9	0.74	[0.46–1.19]	0.18	0.53
rs927650/rs2248137											
CC	21.4	22.9	1.09	[0.80–1.47]	0.59	2.36	30.6	1.62	[1.00–2.60]	0.06	0.23
CG	27.6	31.5	1.21	[0.92–1.59]	0.14	0.58	22.5	0.76	[0.46–1.27]	0.33	1.32
TC	39.5	34.5	0.81	[0.62–1.05]	0.09	0.38	38.2	0.95	[0.61–1.47]	0.88	3.54
TG	11.5	11.1	0.96	[0.65–1.43]	0.81	3.25	8.7	0.73	[0.35–1.56]	0.26	1.05
rs927650/rs2296241											
CA	40.6	41.5	1.04	[0.81–1.34]	0.68	2.74	30.6	0.65	[0.41–1.03]	0.11	0.43
CG	8.4	12.9	1.60	[1.60–2.40]	**0.02**	0.09	22.5	3.14	[1.80–5.49]	$\mathbf{4.0\times10^{-4}}$	$\mathbf{1.6\times10^{-3}}$
TA	13.9	10.9	0.76	[0.51–1.11]	0.10	0.40	11.0	0.77	[0.39–1.51]	0.18	0.73
TG	37.1	34.7	0.90	[0.69–1.17]	0.48	1.92	35.9	0.95	[0.61–1.49]	0.93	3.74
rs927650/rs2248137/rs2296241											
CCA	14.4	10.9	0.72	[0.49–1.06]	0.10	0.50	10.4	0.69	[0.35–1.38]	0.28	1.41
CCG	7.3	11.8	1.71	[1.11–2.63]	**0.02**	0.09	21.1	3.40	[1.90–6.06]	$\mathbf{3.0\times10^{-4}}$	$\mathbf{1.5\times10^{-3}}$
CGA	26.1	30.7	1.25	[0.95–1.65]	0.10	0.50	19.3	0.68	[0.39–1.16]	0.30	1.50
TCG	34.6	32.4	0.91	[0.69–1.18]	0.50	2.49	34.9	1.01	[0.64–1.59]	0.74	3.70
TGA	9.3	8.7	0.93	[0.60–1.44]	0.57	2.86	9.6	1.04	[0.50–2.16]	0.40	1.99

p-Values < 0.05 are boldface. Haplotypes with a frequency (F) > 5% in both groups are listed.
OR, odds ratio; 95% CI, 95% confidence interval.

for the *CYP2R1* (rs12794714, rs10741657), CYP27B1 (rs10877012, rs4646536), and *CYP24A1* (rs927650, rs2248137) polymorphisms. In addition, no differences were observed when stratifying the polymorphisms for gender (data not shown). While the *CYP24A1* rs2296241 polymorphism in the PTC group revealed no difference in the distribution compared with HC, the FTC group showed that the genotype *AA* for the *CYP24A1* rs2296241 polymorphism was less frequent (18.8% vs. 29.5%), whereas the *GG* genotype was more frequent (35.4% vs. 20.5%) in patients with FTC compared with those with HC ($p = 0.05$, corrected [*pc*] = 0.15; Table 1). In addition, the distribution of allele frequencies for the *CYP24A1* rs2296241 polymorphism differed between FTC patients and controls (allele *A*: 41.7% vs. 54.5%, OR 0.6 [95% CI, 0.39–0.92]; allele *G*: 58.3% vs. 45.5%, OR 1.67 [95% CI, 1.08–2.59]; $p = 0.03$, $pc_{trend} = 0.06$). A similar trend was observed in men (data not shown) but after adjusting for multiple comparisons, these associations also were no longer significant.

Haplotype analysis

The LD was calculated between the rs12794714 and rs10741657 polymorphisms within the *CYP2R1* gene (LD: LOD = 56.23; $D' = 0.825$ [0.76–0.88]), between the rs10877012 and rs4646536 polymorphisms within the *CYP27B1* gene (LD: LOD = 158.63; $D' = 0.905$ [0.87–0.93]), and between the following polymorphisms within the *CYP24A1* gene: rs2248137 and rs2296241 (LD: LOD = 67.74; $D' = 0.82$ [0.76–0.87]), rs927650 and rs2248137 (LD: LOD = 14.76; $D' = 0.423$ [0.33–0.5]), and rs927650 and rs2296241 (LD: LOD = 39.07; $D' = 0.534$ [0.47–0.59]).

The haplotype analysis showed that the rare haplotype *rs10877012A/rs4646536T* for *CYP27B1* (1% vs. 3.2%; $pc_{trend} = 0.06$, data not shown) and the haplotype *rs2248137C/rs2296241A* for *CYP24A1* (13.1% vs. 19.1%; $pc = 0.04$) was less frequent in the PTC, whereas the haplotype *rs2248137C/rs2296241G* (56.0% vs. 41.9%; $pc = 0.03$) was more frequent in the FTC compared with the HC. Furthermore, patients with DTC more often carried the haplotypes *rs927650C/rs2296241G* (PTC 12.9% vs. 8.4%, $pc_{trend} = 0.09$; FTC 22.5%, $pc = 1.6\times10^{-3}$) and *rs927650C/rs2248137C/rs2296241G* (PTC 11.8% vs. 7.3%, $pc_{trend} = 0.09$; FTC 21.1%, $pc = 1.5\times10^{-3}$) for *CYP24A1* than the HC (Table 2). Finally, the haplotypes for *CYP2R1* did not reveal any statistically significant associations with DTC (data not shown).

Plasma 25(OH)D_3 and 1,25(OH)$_2D_3$ levels

Data for 25(OH)D_3 and 1,25(OH)$_2D_3$ were available in the plasma of 173 patients with PTC (women $n = 109$; men $n = 64$), 38 patients with FTC (women $n = 24$; men $n = 14$), and 104 patients with HC (women $n = 56$; men $n = 48$).

The median ages of the control subjects differed from those of the patients with DTC. Since one of the possible causes for developing vitamin D deficiency is aging by reduced skin production of the vitamin D precursor, we examined whether a relationship between age in our controls and the production of vitamin D exists. We found no association between age and circulating 25(OH)D_3 or 1,25OH$_2D_3$ levels in our control group (data not shown).

As expected and previously reported (4), the plasma levels of 1,25(OH)$_2D_3$ but not 25(OH)D_3 were significantly lower in individuals with PTC and FTC compared with those with HC ($pc = 3.0\times10^{-7}$ and $pc = 1.8\times10^{-5}$ respectively, data not shown). Twenty and 0.2% of patients were severely vitamin D deficient, 42.3% were deficient, 26% were insufficient, and only 11.5% were sufficient. Nevertheless, only in the HC group but not in the patient group was the vitamin D level

Table 3. $1,25(OH)_2D_3$ Concentration in Patients with Papillary Thyroid Cancer, Follicular Thyroid Cancer, and Healthy Controls According to $25(OH)D_3$ Status

		1,25 $(OH)_2D_3$ [pg/mL]			
25 $(OH)D_3$ status	n	*Median*	*Range*	p	pc
Severely deficient (<10 ng/mL)					
HC	21	43	4–76		
PTC	35	34	7–117	[a]	
FTC	28	28	3–58		
Deficient (10–20 ng/mL)					
HC	44	53	14–93		
PTC	75	35	5–120	**7×10^{-6}**	**2×10^{-5}**
FTC	12	30	14–89	**8.2×10^{-4}**	**2×10^{-3}**
Insufficient (20–30 ng/mL)					
HC	27	68	33–92		
PTC	39	49	3–86	**1×10^{-3}**	**3×10^{-3}**
FTC	3	45	8–47	**0.03**	0.09
Sufficient (>30 ng/mL)					
HC	12	58	38–72		
PTC	24	33	8–96	**2×10^{-3}**	**6×10^{-3}**
FTC	11	51	25–67	0.10	0.30

p-Values <0.05 are boldface. Kruskal–Wallis test global $p<0.05$; multiple Conover–Iman comparison (Bonferroni corrected).
[a]Kruskal *p* global $p>0.05$.
$1,25(OH)_2D_3$, 1,25-hydroxyvitamin; $25(OH)D_3$, 25-hydroxyvitamin D_3.

significantly reduced in winter compared with the summer season [$1,25(OH)_2D_3$: 43 pg/mL vs. 56 pg/mL, $p=0.04$; $25(OH)D_3$: 12 ng/mL vs. 20 ng/mL, $p=0.01$]. In contrast, patients with DTC had similarly low levels of $25(OH)D_3$ during winter and summer periods (PTC: 16 ng/mL vs. PTC: 17 ng/mL, $p=0.78$; FTC: 14 ng/mL vs. 12 ng/mL, $p=0.87$).

In order to evaluate the effect of $25(OH)D_3$ status on the production of $1,25(OH)_2D_3$ levels, the patients and controls were divided into four $25(OH)D_3$ subgroups (severely deficient, deficient, insufficient, and sufficient). While $25(OH)D_3$-deficient PTC and FTC patients have a lower concentration of $1,25(OH)_2D_3$ in median (35 and 30 pg/mL) than controls (53 pg/mL; $pc=2\times10^{-5}$, $pc=2\times10^{-3}$, respectively), no difference was observed in the severely deficient group. Furthermore, only PTC patients with an insufficient or sufficient $25(OH)D_3$ had lower levels of $1,25(OH)_2D_3$ compared with the controls (Table 3).

Plasma 1,25(OH)$_2$D$_3$ levels and genotypes

In order to analyze whether the haplotype combination of *CYP27B1* and *CYP24A1* was associated with higher or lower vitamin D levels [$25(OH)D_3$ and $1,25(OH)_2D_3$], the genotypes between patients with DTC and HC as well as within the groups were compared. A relationship between $1,25(OH)_2D_3$ (Table 4) but not $25(OH)D_3$ status (data not shown) with polymorphisms of the vitamin D system genes was observed. Patients with PTC and the genotype *CC/AC* of rs10877012, genotypes *CC/CG* of rs2248137 and genotypes *AA/AG* of rs2296241 had lower levels of $1,25(OH)_2D_3$ in comparison to those with HC with the same combinations. In contrast, all three genotypes of the rs4646536 and rs927650 polymorphisms

Table 4. Distribution of the *CYP27B1* (rs10877012, rs4646536) and *CYP24A1* (rs927650, rs2248137, rs2296241) Polymorphisms According to the Median $1,25(OH)_2D_3$ Plasma Levels in Patients with Papillary Thyroid Cancer, Follicular Thyroid Cancer, and Healthy Controls

	1,25$(OH)_2D_3$ (median; pg/mL)							
	HC, n=*104*		*PTC*, n=*173*			*FTC*, n=*38*		
	Median (n)	*Range*	*Median* (n)	*Range*	pc	*Median* (n)	*Range*	pc
rs10877012								
CC	52 (47)	21–93	37 (79)	5–96	**3.0×10^{-4}**	46 (22)	3–67	0.15
AA	47 (13)	14–88	31 (20)	3–65	0.09	14 (3)	13–28	0.08
AC	50 (44)	23–91	39 (74)	4–120	**2.9×10^{-4}**	31 (13)	8–89	**6.0×10^{-3}**
rs4646536								
TT	51 (49)	25–93	36 (80)	5–96	**4.8×10^{-5}**	46 (22)	3–67	0.12
CC	47 (13)	29–88	27 (19)	3–65	**8.8×10^{-3}**	14 (3)	13–28	**0.04**
TC	51 (42)	14–91	39 (74)	3–67	**5.8×10^{-3}**	31 (13)	8–89	**0.01**
rs927650								
TT	57 (34)	23–91	39 (40)	3–80	**3.4×10^{-4}**	39 (8)	18–57	0.06
CC	47 (29)	25–86	36 (48)	7–117	**0.01**	36 (11)	3–67	0.44
CT	47 (41)	14–93	36 (85)	4–120	**5.9×10^{-3}**	35 (19)	8–89	0.07
rs2248137								
CC	54 (43)	21–86	37 (68)	3–96	**1.2×10^{-5}**	41 (14)	14–89	0.18
GG	54 (14)	29–93	37 (28)	4–69	0.13	28 (10)	3–67	0.12
CG	48 (47)	14–92	37 (77)	5–120	**5.0×10^{-3}**	34 (14)	8–60	**0.04**
rs2296241								
AA	58 (31)	29–93	38 (49)	5–117	**6.0×10^{-5}**	41 (6)	3–55	0.26
GG	49 (24)	23–86	39 (44)	7–87	0.19	20 (13)	8–60	**0.01**
AG	48 (49	14–92	35 (80)	3–120	**3.7×10^{-4}**	38 (19)	13–89	0.46

p-Values <0.05 are boldface. Kruskal–Wallis test global $p<0.05$; multiple Conover–Iman comparison (Bonferroni corrected).

demonstrated a lower level of $1,25(OH)_2D_3$ in patients with PTC compared with those with HC. In addition, a lower level of circulating $1,25(OH)_2D_3$ was observed in patients with FTC of rs10877012 genotype *AC*, rs4646536 genotypes *CC/TC*, rs2248137 genotype *CG*, and rs2296241 genotype *GG* (Table 4).

$1,25(OH)_2D_3$ production according to $25(OH)D_3$ status and genotypes

The distribution of the different polymorphisms within the four $25(OH)D_3$ categories (severely deficient, deficient, insufficient, and sufficient) was not different between cases and controls (data not shown). However, the genotypes GG for *CYP24A1* rs2296241 and AC for *CYP27B1* rs10877012 were more frequent (100% vs. 22.2%, $p = 0.02$, $pc = 0.06$ and 100% vs. 25.9%, $p = 0.04$, $pc = 0.12$, respectively) in the $25(OH)D_3$ insufficient group and, subsequently, had a lower $1,25(OH)_2D_3$ production than HC. These differences did not remain significant after correction of the *p*-values.

Discussion

Higher levels of vitamin D may reduce the risk of several cancers, possibly through the genomic effects modulated by the VDR, and the autocrine/paracrine effects of the VDR ligand, $1,25(OH)_2D_3$. Vitamin D and its potential relevance to cancer prevention has become evident by the discovery that the cell types not involved with calcium metabolism are abundantly endowed with vitamin D system enzymes CYP2R1, CYP27B1, VDR, and CYP24, which produce, respond to, and degrade $1,25(OH)_2D_3$ (15–17). On the other hand, there are vitamin D response elements (VDREs) with regard to more than 200 genes that appear to be modulated by $1,25(OH)_2D_3$ and, to a lesser extent, by 25(OH)D, influencing cell functions in a cell- and tissue-specific manner (18,19).

The cascade of enzymatic reactions that lead to the biosynthesis of $1,25(OH)_2D_3$ is complex and requires the participation of many gene products. It is conceivable that polymorphisms in any of the several genes (e.g., *CYP2R1*, *CYP27B1*, and *CYP24A1*) in this pathway could lead to differences in endogenous biosynthesis and bioavailability of $1,25(OH)_2D_3$. Genetic association studies investigating the role of vitamin D in colon, breast, and prostate cancers have primarily focused on polymorphisms within the VDR gene (20,21) with limited data available for other genes in the vitamin D pathway, including enzymes that activated and deactivated vitamin D (22–29). Based on these observations and the fact that an association between VDR polymorphisms and DTC risk was previously described in our group (4), we investigated in the present case-control study (all Germans) whether polymorphisms within the *CYP2R1* (rs12794714, rs10741657), *CYP27B1* (rs10877012, rs4646536), and *CYP24A1* (rs927650, rs2248137, rs2296241) genes could play a role in the susceptibility to DTC.

Similar to other studies, which tested the same sequence variants (rs10877012, rs4646536) within the *CYP27B1* gene in different types of cancer, we found no significant differences in our case-control comparison (22,25,26). However, the haplotype analysis suggests that the rare haplotype *rs10877012A/rs4646536T* within the *CYP27B1* gene may be protective against PTC. To date, 3 out of 12 discovered polymorphisms of the *CYP27B1* gene, studied in various cancers (22,24,25–29) (rs8176345, rs4646537, rs3782130), were reported to be associated with prostate cancer risk alone (26,29). Furthermore, our finding regarding polymorphisms within the *CYP24A1* gene are consistent with a previous analysis in which no significant differences in the genotype distributions between cancer patients and controls with regard to the rs927650 (24,27,28), the rs2248137 (25), and the rs2296241 polymorphisms were found (24,26–28). However, Holt *et al.* (29) observed significantly altered risks of recurrence/progression and cancer death in relation to rs927650 and rs2296241 *CYP24A1* polymorphisms in patients with prostate cancer. The genotype and allele analysis revealed a trend association between the *CYP24A1* rs2296241 polymorphism and FTC in our study. This indicates that the presence of the *CYP24A1* rs2296241 allele could contribute to the development of DTC. Effectively, the haplotype combination containing rs2296241 alleles showed that the haplotype *rs2248137C/rs2296241A* protects against PTC, while the haplotypes *rs927650C/rs2296241G* and *rs927650C/rs2248137C/rs2296241G* for *CYP24A1* appear to be associated in DTC (weak in PTC and strong in FTC) with an increasing risk. The three investigated polymorphisms within the *CYP24A1* gene are intronic (rs927650 and rs2248137) and not evolutionarily conserved with the exception of rs2296241 (conservation score 0.99), a synonymous polymorphism in exon 4. This result is important for several reasons: First, a recent study revealed that the promoter region of the *CYP24A1* gene contains VDREs and, thus, emphasized the special role of the *CYP24A1* gene in $1,25(OH)_2D_3$ signaling, which is affected by polymorphism (30). The functional effects of the *CYP24A1* polymorphisms investigated here remain unclear, but several novel polymorphisms have recently been identified in the promoter region 5′ of exon 1 in the *CYP24A1* gene that exerted a functional impact on VDRE binding and transactivation *in vitro* and altered expression of *CYP24A1 in vivo* (31). The rs2296241 is located near this region and could be linked to this functional polymorphism. This could explain an association with DTC. Second, the extra renal expression of *CYP27B1* in a wide variety of nonclassical target tissues includes the thyroid epithelium where $25(OH)D_3$ can be converted to $1,25(OH)_2D_3$, affecting the differentiation, proliferation, and apoptosis of cells (32). *CYP24A1* mRNA expression is up-regulated in tumors, and may counteract $1,25(OH)_2D_3$ antiproliferative activity, presumably by decreasing $1,25(OH)_2D_3$ levels (15,33,34). In this context, decreased $1,25(OH)_2D_3$ concentration in the peripheral blood of patients with DTC ($n = 147$) was previously reported by our group (4). The present study confirms the previous results of our group (4) describing lower $1,25(OH)_2D_3$ levels in DTC patients. In extension to that, the present study provides additional information about calcium and creatinine levels. These findings have been confirmed by others (35). However, the level of $25(OH)D_3$ was not statistically different from HC (4); the majority of the patients as well as controls had deficient levels of $25(OH)D_3$. In probands with deficient $25(OH)D_3$ status, the differences in the $1,25(OH)_2D_3$ levels between patients and controls were more distinct.

The difference in the $25(OH)D_3$ status/$1,25OH_2D_3$ production between controls and patients could be attributed to the age of the patients. However, since there is no correlation between age and vitamin in the control group, it is likely that the differences are due to the disease.

Since lower levels of $1,25(OH)_2D_3$ were measured in our patient groups compared with HC, an overexpression of the *CYP24A1* in DTC tissues can be expected. An increased expression of this enzyme in the thyroid tissue of patients with DTC and in thyroid cancer cell lines has been recently documented (33,34). Finally, we found that the median plasma concentration of $1,25(OH)_2D_3$ but not $25(OH)D_3$ differs significantly depending on the genotypes. Subsequently, the risk alleles in the haplotype combination *rs927650C/rs2248137C/rs2296241G* for *CYP24A1* in DTC are associated with a lower $1,25(OH)_2D_3$ concentration. This may explain that polymorphisms in the *CYP24A1* gene could influence the production of $1,25(OH)_2D_3$ possibly by an altered balance of *CYP27B1* and *CYP24* expression.

A recent report showed the active $1,25(OH)_2D_3$ compound as well as a superagonistic VDR ligand to be effective in anaplastic thyroid carcinoma in conjunction with paclitaxel or suberoylanilide hydroxamic acid (36). This illustrates the potential of vitamin D signaling in undifferentiated carcinoma and may extend to DTC.

Our results suggest that haplotypes within the *CYP24A1* gene, low circulating $25(OH)D_3$ levels (deficiency), and a reduced conversion to $1,25(OH)_2D_3$ enhance the risk to DTC. Whether deficient $25(OH)D_3$ levels in combination with the genetic variants lead to lower circulating $1,25(OH)_2D_3$, and thereby to a depressed vitamin D action, should be evaluated in future studies in DTC.

To our knowledge, this is the first study that evaluates both plasma vitamin D status and polymorphisms in the genes encoding enzymes that are involved in the metabolism of vitamin D_3 in relation to DTC. The frequent vitamin D deficiency in patients with DTC warrants clinical studies with supplementation that may have secondary preventive action.

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Disclosure Statement

The authors declare that no competing financial interests exist.

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