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ORIGINAL ARTICLE

Association between vitamin D receptor genetic polymorphisms and acute cellular rejection in liver-transplanted patients

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Summary

Vitamin D receptor (*VDR*) polymorphisms may confer susceptibility to immunologically mediated liver diseases. We aimed to verify whether recipient *VDR* polymorphisms might affect the incidence of acute cellular rejection (ACR) of the graft after liver transplantation (LT). Two hundred and fifty-one liver-transplanted patients surviving at least 1 month were studied. ACR in the first post-LT year was graded according to the Banff score. Recipients genotyping for VDR polymorphic sites (FokI C>T, BsmI G>A, ApaI T>G, TaqI T>C) was performed. A significant difference was found between patients with and without ACR episodes in allele frequencies of BsmI (G: 0.660 vs. 0.545, $P = 0.017$) and TaqI (T: 0.667 vs. 0.543, $P = 0.010$). Patients carrying the G-*-T/G-*-T diplotypes of the BsmI G>A, ApaI T>G and TaqI T>C experienced more frequently ACR: 33/79 Vs 42/172, $P = 0.005$. Carriage of G-*-T/G-*-T diplotypes was an independent predictor of ACR (OR 2.41, $P = 0.006$), with CMV reactivation (OR 2.34, $P = 0.033$) and HCV aetiology (OR 1.86, $P = 0.036$). In conclusion, recipient *VDR* polymorphic loci are strongly associated with ACR occurrence during the first year after LT. The knowledge of *VDR* genetic polymorphisms may be helpful in identifying recipients at higher risk of ACR and in selecting them for a more aggressive immunosuppressive therapy.

Introduction

The incidence of acute cellular rejection (ACR) following liver transplantation (LT), initially reported to range from 30% to 70% in the first year, has been found to decrease in the more recent series [1]. Single ACR episodes do not seem to affect the good long-term function of the liver allograft [2]; while recurrent episodes may result in permanent graft damage [3], ACR is effectively prevented by using calcineurin inhibitors (CNI), either cyclosporine [4] or tacrolimus [5]. Unfortunately, CNI administration is associated with adverse effects, such as renal failure, diabetes mellitus, hypertension, neurotoxicity and hyperlipidaemia [6]. Therefore, researchers had tried to identify ACR predictors, hoping to tailor the immune-suppressive regimen to the needs of individual patients. Several such predictors, including age and ethnicity of recipients, aetiology of liver disease, cytomegalovirus (CMV) reactivation and others, have indeed been identified [7–9].

The inflammatory environment in which the recipient immune system reacts with graft antigens seems to play a pivotal role in determining the outcome of engraftment

[10]. Thus, genes coding for molecules related to the immune response have been explored as potential ACR modulators, including HLA matches [11] and cytokine gene polymorphisms involving the interleukin-6 (IL-6) [12], interferon-γ [12] and CTLA-4 [13]. In fact, it is accepted that type and level of different cytokines may strongly influence the balance between rejection and tolerance [10].

The vitamin D receptor (*VDR*) is a member of the nuclear receptor super-family of ligand-inducible transcription factors involved in many physiological processes, including cell growth and differentiation, embryonic development and metabolic homoeostasis. The transcriptional activity of this receptor is modulated by ligands such as steroids, retinoids and other lipid-soluble compounds and by nuclear proteins acting as co-activators and co-repressors [14,15]. The ligand *VDR* heterodimerizes with the retinoid X receptor and binds to vitamin D response elements in the promoter region of target genes, thereby affecting target genes transcription. The *VDR* gene itself is quite large (over 100 kb) and has an extensive promoter region capable of generating multiple tissue-specific transcripts [16]. Among the numerous *VDR* polymorphisms described, the more investigated were FokI C>T (rs2228570) and TaqI T>C (rs731236) polymorphic sites on the coding sequence, BsmI G>A (rs1544410) and ApaI T>G (rs7975232) on the last intron [16]. The genotypic combinations of these polymorphisms are believed to alter the immune response [17]. In fact, these *VDR* polymorphisms have been associated with several autoimmune diseases, such as primary biliary cirrhosis [18–22], auto-immune hepatitis [18], type 1 diabetes mellitus [23] and multiple sclerosis [24]. Furthermore, *VDR* polymorphisms have been related to the development of cancer in several organs, including breast, prostate, skin, colon-rectum, bladder and kidney, although with conflicting observations [25–27].

The aims of the present study were to investigate whether (i) the aforementioned *VDR* polymorphisms, in liver tranplant recipients, may be associated with ACR and (ii) to verify if a specific risk diplotype could be identified.

Materials and methods

Patients

Two hundred fifty-one consecutive adult patients who underwent LT between 1996 and 2008 at our institution were studied. The inclusion criteria were survival for at least 1 month after LT, availability of a stored blood sample obtained at the time of listing and informed consent to participate in the study. The main clinical and demographic characteristics of donors and recipients are presented in Table 1. All the patients were maintained on an immunosuppressive regimen that was either cyclosporine- ($n = 77$) or tacrolimus-based ($n = 174$), used, in the first few months after LT, in association with corticosteroids. Cyclosporine dosage was calculated to obtain predose serum levels ranging from 250 to 350 μg/l in the first 6 weeks after transplant and from 50 to 150 μg/l thereafter. Tacrolimus dosage was calculated to obtain predose serum levels ranging from 10 to 15 μg/l in the first 6 weeks after transplant and from 5 to 10 μg/l thereafter. CNI serum concentration was recorded at day 1, after the initial loading dose, and thereafter at the following days: 15, 30, 60, 90, 120, 150, 180 and 360. The CNI mean daily concentration was estimated calculating the area under the curve (AUC) by the trapezoid rule. Corticosteroid therapy was started at the transplant operation with an i.v. bolus of 500 mg of methylprednisolone, followed by 250 mg on the second and third days after transplant. Starting from the fourth day after transplant, a dose of 40 mg of prednisone was administered orally and tapered discretionally by the single physician with a schedule aimed to complete steroid withdrawal by the end of the fourth postoperative month. In the event of moderate-to-severe ACR, intravenous methyl-prednisolone boluses (1 g daily) were administered for three consecutive days, followed by oral prednisone, 40 mg for four further days [28].

Histology

Biopsy specimens were evaluated by experienced transplant pathologists to determine liver allograft rejection scores. All the biopsy specimens showing ACR were re-evaluated by a single expert pathologist (C.A.) and scored according to the Banff scheme [29]. In this system, portal inflammation, bile duct damage and venous endothelial inflammation are each graded semi-quantitatively on a scale ranging from 0 (absent) to 3 (severe). The individual scores are added to produce an overall rejection score ranging from 0 to 9. For the purposes of this study, only moderate-to-severe (Banff score ≥5) rejection episodes were considered; these were searched on all the available liver biopsies (both per protocol liver biopsies performed at 1, 6 and 12 months after LT and on-demand liver biopsies) performed during the first year after LT.

Molecular biology studies

Genomic DNA was isolated from whole blood using the QIAamp DNA blood mini kit (Qiagen-Milan-Italy) according to the manufacturer's instructions. Four polymorphisms of *VDR* were genotyped: FokI C>T (rs10735810) and TaqI T>C (rs10735810) polymorphic

Table 1. Main demographic and clinical characteristics of the studied population. Data are presented as a whole and in relationship with the carriage of G-*-T/G-*-T diplotypes. Continuous variables are reported as medians (range) or mean ± SD and categorical variables as frequencies (%). The statistical analysis was performed by means of the Student's *t*-test (continuous variables) and Pearson chi-square test (categorical variables).

	Total (*n* = 251)	G-*-T/G-*-T carriers (*n* = 79)	G-*-T/G-*-T not carriers (*n* = 172)	*P*
Recipient male gender	182 (72.5)	54 (68.4)	128 (74.4)	0.318
Donor male gender	169 (67.3)	48 (60.8)	121 (70.3)	0.133
Recipient age (years)	55 (22–68)	55.9 ± 7.7	52.3 ± 8.4	0.001
Donor age (years)	47 (11–81)	49.3 ± 16.7	45.5 ± 17.1	0.105
Recipient BMI (kg/m^2)	25.0 (14.8–48.5)	25.1 ± 3.2	25.1 ± 3.9	0.917
Aetiology of liver disease				
HBV	37 (14.7)	6 (7.6)	31 (18.0)	0.030
HCV	101 (40.2)	27 (34.2)	74 (43.0)	0.184
Alcoholic	80 (31.9)	31 (39.2)	49 (28.5)	0.090
Other	28 (11.2)	14 (17.7)	14 (8.1)	0.025
FHF	5 (2.0)	1 (1.3)	4 (2.3)	0.577
MELD score at transplant	14 (6–40*)	15 ± 6	15 ± 7	0.900
Presence of DM at transplant	73 (29.1)	26 (32.9)	47 (27.3)	0.366
Presence of HCC	82 (32.7)	33 (41.8)	49 (28.5)	0.037

BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; FHF, fulminant hepatic failure; DM, diabetes mellitus; HCC, hepatocellular carcinoma.
*Two of five patients with FHF had a MELD score = 40.

sites in the coding sequence, BsmI G>A (rs1544410) and ApaI T>G (rs7975232) in the last intron. For the detection of the *VDR* polymorphisms, the polymerase chain reaction (PCR) technique followed by fragment restriction assays (RFLP) was applied. The PCR amplifications were carried out in a total volume of 10 μl containing 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 0.01% tween-20, 0.2 mmol/l deoxyribonucleotides, 24 pmol of each primer, 2.0 mmol/l $MgCl_2$, 0.5 U hot-start Taq DNA polymerase (RighTaq-Euroclone-Milan-Italy). The sequences of primers used for FokI were: (*f*) 5′-TGCA GCCTTCACAGGTCATA-3′, (*r*) 5′-GGCCTGCTTGCTGT TCTTAC-3′; for TaqI and ApaI were (*f*) 5′-ACGTCTGCA GTGTGTTGGAC-3′, (*r*) 5′-TCACCGGTCAG CAGTCA TAG-3′ and those used for BsmI were (*f*) 5′-CAGTTCAC GCAAGAGCAGAG-3′, (*r*) 5′-ACCTGAAGG GAGACG TAGCA-3′. All the primers were newly designed using NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The cycling conditions for all *VDR* polymorphisms were set as: 40 cycles at 95 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min. In a total volume of 20 μl, amplified DNA (10 μl) was digested overnight with 2 U of restriction endonucleases using the buffers and temperatures recommended by the manufacturers. The FokI C>T polymorphism was analysed by digestion of a 157 bp PCR product with FokI (New England Biolabs-Hitchin-UK), which resulted in two fragments of 121 and 36 bp in the presence of the T allele and in an uncut fragment in the presence of the C allele. The TaqI T>C and ApaI T>G polymorphisms were analysed by digestion of a 211 bp PCR product with TaqI (New England Biolabs-Hitchin-UK), which resulted in two fragments of 172 and 39 bp in the presence of the C allele and by digestion with ApaI (New England Bioloabs-Hitchin-UK), which resulted in two fragments of 121 and 90 bp for the G allele. The BsmI G>A polymorphism was analysed by digestion of a 236 bp PCR product with BsmI (New England Biolabs-Hitchin-UK), which resulted in two fragments of 197 and 39 bp in the presence of the G allele. All PCR reactions were carried out in a Techne TC-412 thermal cycle and PCR products were sized by electrophoresis on a 3% agarose gel stained with ethidium bromide.

Statistical analysis

Statistical analysis of data was performed using the BMDP dynamic statistical software package 7.0 (Statistical Solutions, Cork, Ireland). The measures used throughout the study to indicate central tendency and dispersion in continuous variables were medians (range) or mean ± standard deviation. Categorical variables are presented as frequencies (%). Differences between groups were assessed by the Student's *t*-test (continuous variables) and the Pearson chi-square test (categorical variables). The chi-square G test ''Goodness of Fit'' was employed to verify whether the proportions of the four polymorphisms were distributed in accordance with the Hardy-Weinberg equation. The existence of differences in allelic and genotypic frequencies between different groups was assessed by means of Pearson chi-square test or chi-square test for linear trend when appropriate and calculating the odds ratio (OR) with the 95% confidence intervals (CI). Haplotype reconstruction from population genotype data and inferred phased diplotype calculation for each transplanted patient were performed using ARLEQUIN integrated software package for population genetics, version

3.1. Analysis of molecular variance (AMOVA) with a global approach was performed to assess whether haplotype allelic content differed among groups. Locus-by-locus AMOVA was utilized to assess the statistical contribution given by each polymorphism. Linkage disequilibrium among the four analysed *VDR* polymorphic loci in the studied patients was determined using the Haploview software. Stepwise logistic regression analysis with a forward approach was used to verify whether the absence or presence of specific *VDR* diplotypes could be considered an independent predictor of ACR. Time-to-event analysis was performed to assess the influence of *VDR* diplotypes in the occurrence of ACR using the Mantel–Cox test. The Cox proportional Hazard model was used to verify whether *VDR* diplotypes could be considered independent predictors of ACR occurrence.

Results

Acute cellular rejection

During the first year post LT, 93 ACR episodes were recorded. Seventy five (29.9%) patients had at least one episode of moderate-to-severe (Banff score ≥5) ACR. Sixty-three patients had a single episode, 12 patients had two episodes and two patients had three episodes of ACR. Sixty-five of 93 (69.9%) ACR episodes occurred in the first trimester after LT. In all these cases, rejection was controlled by steroid boluses. Table 2 reports the associations between occurrence of ACR and pertinent demographic and clinical variables. A significant association was observed only between CMV reactivation and ACR occurrence.

VDR polymorphisms and ACR

Recipient *VDR* genotypic frequencies were the following: FokI C/C = 109 (43.4%), C/T = 113 (45.0%), T/T = 29 (11.6%); BsmI G/G = 82 (32.7%), G/A = 127 (50.6%), A/A = 42 (16.7%); ApaI T/T = 82 (32.7%), T/G = 130 (51.8%), G/G = 39 (15.5%); TaqI. T/T = 81 (32.3%), T/C = 129 (51.4%) and C/C = 41 (16.3%). The corresponding allelic frequencies were FokI C = 0.659 and T = 0.341; BsmI G = 0.580 and A = 0.420; ApaI T = 0.586 and G = 0.414; TaqI T = 0.580 and C = 0.420. All the four genotypic frequencies observed did not differ from what were expected according to the Hardy–Weinberg formula ($P > 0.20$). Table 3 displays the association among the four *VDR* polymorphism allele frequencies and the occurrence of at least one ACR episode, showing that carriage of the minor BsmI A allele and of TaqI C allele protected from ACR episodes. Table 4 displays the associations among the four *VDR* polymorphism genotype frequencies and the occurrence of at least one ACR episode. Considering both dominant and additive models for the minor allele, BsmI and TaqI were associated strongly with a lower occurrence of ACR. Global AMOVA demonstrated a significant difference in haplotype frequencies (Table 5) between the two groups of patients ($P = 0.012$), with or without ACR episodes. When locus-by-locus approach was performed, an even more significant difference was detected ($P = 0.002$). BsmI ($P = 0.019$), ApaI ($P = 0.046$) and TaqI ($P = 0.005$) significantly differed between the two group of patients, while this was not evident for FokI ($P = 0.125$). A significant linkage disequilibrium was observed among BsmI,

Table 2. Main demographic and clinical variables in relationship with the occurrence of at least one moderate/severe (Banff ≥5) rejection episode within the first year after LT. The statistical analysis was performed by means of the Pearson chi-square test.

	Moderate/severe rejection episodes		
	Yes ($n = 75$)	No ($n = 176$)	*P*
Recipient male gender	54 (72.0%)	128 (72.7%)	0.906
Donor male gender	50 (66.7%)	119 (67.6%)	0.884
Recipient age >55 years	34 (45.3%)	88 (50.0%)	0.498
Donor age >45 years	44 (58.7%)	92 (52.3%)	0.352
Recipient BMI >25 kg/m^2	35 (46.7%)	86 (48.9%)	0.750
HCV positive recipients	35 (46.7%)	66 (37.5%)	0.175
LT UNOS MELD score >15	28 (37.3%)	74 (42.0%)	0.487
Presence of HCC	21 (28.0%)	61 (34.7%)	0.303
Presence of DM at LT	22 (29.3%)	51 (29.0%)	0.955
Cumulative steroid dose >1250 mg	48 (64.0%)	111 (63.1%)	0.889
TAC-based immune-suppression	49 (65.3%)	125 (71.0%)	0.371
Cold ischaemia time >12 hours	1 (1.3%)	9 (5.1%)	0.161
CMV infection	23 (30.7%)	32 (18.2%)	0.029

HCV, hepatitis C virus; UNOS MELD, united nation organ sharing model of end stage liver disease; HCC, hepatocellular carcinoma; LT, liver transplantation; DM, diabetes mellitus; TAC, tacrolimus; CMV, cytomegalovirus.

	Moderate/severe rejection episodes				
VDR	Yes (n = 75)	No (n = 176)	OR	95% CI	*P*
FokI	C = 0.713	C = 0.636	1	Reference	
	T = 0.287	T = 0.364	0.703	0.465–1.064	0.096
BsmI	G = 0.660	G = 0.545	1	Reference	
	A = 0.340	A = 0.455	0.618	0.416–0.919	0.017
ApaI	T = 0.520	T = 0.614	1	Reference	
	G = 0.480	G = 0.386	1.466	0.998–2.154	0.051
TaqI	T = 0.667	T = 0.543	1	Reference	
	C = 0.333	C = 0.457	0.593	0.398-0.883	0.010

Table 3 Allelic frequencies of the *VDR* FokI C>T (rs10735810), BsmI G>A (rs1544410), ApaI T>G (rs7975232) and TaqI T>C (rs10735810) polymorphisms in relationship with the occurrence of at least one moderate/severe (Banff ≥5) rejection episode within the first year after LT. The ORs were constructed considering as reference the wild type for each polymorphism. The statistical analysis was carried out using the Pearson chi-square test.

VDR, vitamin D receptor; LT, liver transplantation; OR, odds ratio; CI, confidence interval.

Table 4. Genotype frequencies of *VDR* FokI C>T (rs10735810), BsmI G>A (rs1544410), ApaI T>G (rs7975232) and TaqI T>C (rs10735810) polymorphisms in relationship with the occurrence of at least one moderate/severe (Banff ≥5) rejection episode within the first year after LT. The odds ratios were constructed considering as reference the wild type for each polymorphism. The statistical analysis was carried out using the Pearson chi-square test. Results for the dominant, recessive and additive genetic models are presented in the footnote.

	Moderate/severe rejection episodes				
VDR	Yes (n = 75)	No (n = 176)	OR	95% CI	*P*
FokI	C/C = 36 (48.0)	C/C = 73 (41.5)	1	Reference	
	C/T = 35 (46.7)	C/T = 78 (44.3)	0.910	0.519–1.596	0.743
	T/T = 4 (5.3)	T/T = 25 (14.2)	0.324	0.110–0.965	0.042
BsmI	G/G = 33 (44.0)	G/G = 49 (27.8)	1	Reference	
	A/G = 33 (44.0)	A/G = 94 (53.4)	0.521	0.289–0.941	0.030
	A/A = 9 (12.0)	A/A = 33 (18.8)	0.405	0.174–0.965	0.036
ApaI	T/T = 20 (26.7)	T/T = 62 (35.2)	1	Reference	
	G/T = 38 (50.7)	G/T = 92 (52.3)	1.280	0.685–2.391	0.441
	G/G = 17 (22.7)	G/G = 22 (12.5)	2.395	1.075–5.343	0.032
TaqI	T/T = 34 (45.3)	T/T = 47 (26.7)	1	Reference	
	C/T = 32 (42.7)	C/T = 97 (55.1)	0.456	0.252–0.825	0.009
	C/C = 9 (12.0)	C/C = 32 (18.2)	0.389	0.167–0.909	0.029

	Genetic models						
	Dominant model for minor allele			Recessive model for minor allele			Additive model
	OR	95% CI	*P*	OR	95% CI	*P*	*P*
FokI	0.768	0.447–1.319	0.340	0.340	0.119–0.974	0.044	0.095
BsmI	0.491	0.280–0.859	0.012	0.591	0.272–1.288	0.190	0.015
ApaI	1.496	0.826–2.706	0.186	2.052	1.026–4.106	0.042	0.044
TaqI	0.439	0.251–0.770	0.004	0.614	0.282–1.340	0.225	0.008

VDR, vitamin D receptor; LT, liver transplantation; OR, odds ratio; CI, confidence interval.

ApaI and TaqI, with a strong association between BsmI and TaqI (r^2 0.93, Fig. 1). Thus, FokI was excluded from further analysis concerning diplotypes.

VDR diplotypes and ACR

Patients carrying the diplotypes obtained combining the G-G-T and G-T-T haplotypes (n = 79) experienced more frequently ACR episodes than the remaining patients (n = 172): 33/79 vs. 42/172, P = 0.005. At stepwise logistic regression analysis (covariates variables listed in Table 2), the carriage of the G-*-T/G-*-T diplotypes (OR 2.42, 95% CI 1.34–4.32, P = 0.006) was found to be an independent predictor of ACR occurrence, in association with CMV infection (OR 2.34, 95% CI 1.21–4.52, P = 0.033) and HCV aetiology of liver disease (OR 1.86, 95% CI 1.04–3.36, P = 0.036). At the time-to-event analysis, carriage of the G-*-T/G-*-T diplotypes was significantly related to both earlier and more common occurrences of ACR episodes (Fig. 2). By means of a

Table 5. Inferred haplotype frequencies of the VDR *VDR* FokI C>T (rs10735810), BsmI G>A (rs1544410), ApaI T>G (rs7975232) and TaqI T>C (rs10735810) polymorphisms in relationship with the occurrence of at least one moderate/severe (Banff ≥5) rejection episode within the first year after LT.

	Moderate/severe rejection episodes	
VDR haplotypes	Yes (n = 75)	No (n = 176)
T-G-T-T	3 (2.0%)	20 (5.7%)
C-A-T-C	41 (27.3%)	125 (35.5%)
C-G-G-T	41 (27.3%)	56 (15.9%)
T-G-G-T	31 (20.7%)	78 (22.2%)
C-G-T-T	24 (16.0%)	34 (9.6%)
T-A-T-C	9 (6.0%)	30 (8.5%)
C-A-T-T	1 (0.7%)	3 (0.9%)
C-G-T-C	0 (0.0%)	4 (1.1%)
C-A-G-C	0 (0.0%)	2 (0.6%)

VDR, vitamin D receptor; LT, liver transplantation.

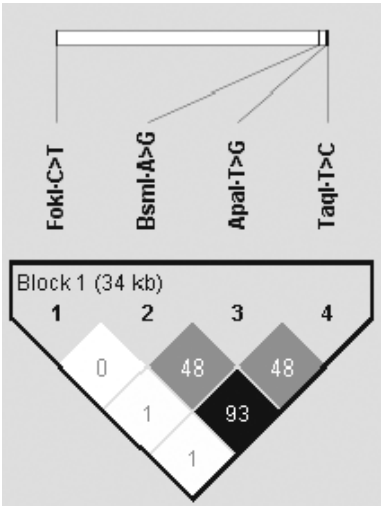

Figure 1 Schematic representation of linkage disequilibrium in the studied population (n = 251) among the four VDR polymorphisms: FokI C>T (rs10735810), BsmI G>A (rs1544410), ApaI T>G (rs7975232) and TaqI T>C (rs10735810). R^2 for linkage disequilibrium between each marker is reported. Shades of grey are in proportion to the R^2 value, expressing the strength of the linkage disequilibrium.

multivariate approach (Cox proportional Hazard model, covariates variables listed in Table 2), the association between the carriage of G-*-T/G-*-T diplotypes and the occurrence of ACR was confirmed to be significant (P = 0.009), as it was that with CMV (P = 0.046) and HCV aetiology of liver disease (P = 0.034). The mean daily concentration of cyclosporin was 173 ± 42 (mean ± standard deviation) ng/ml, whereas that of tacrolimus was 8.59 ± 1.72. No significant difference in the CNI mean daily concentrations was detected according to the carriage of G-*-T/G-*-T diplotypes, either for cyclosporin (G-*-T/G-*-T diplotypes = 169 ± 44 ng/ml vs. all other diplotypes = 175 ± 42, P = 0.61) or for tacrolimus (G-*-T/G-*-T diplotypes = 8.68 ± 1.67 ng/ml vs. all other diplotypes = 8.54 ± 1.74, P = 0.62).

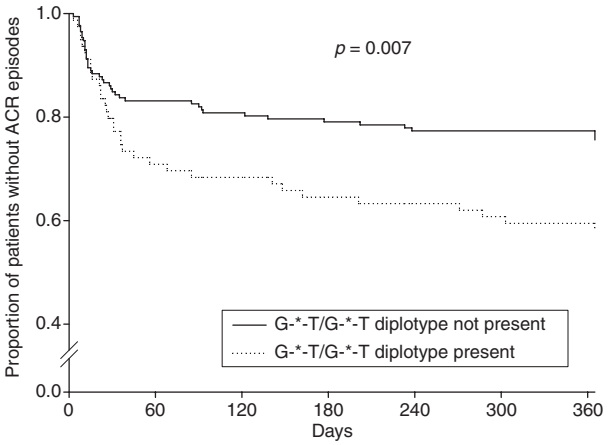

Figure 2 Time-to-event analysis in the occurrence of at least one moderate-to-severe acute cellular rejection (ACR) episode during the first year after liver transplantation. Recipient were stratified according to the carriage (n = 79) or not (n = 172) of G-*-T/G-*-T diplotypes. The statistical analysis was performed using the Mantel–Cox test for linear trend.

Discussion

The outcome of engraftment depends on the characteristics of the inflammatory environment in which donor-reactive $CD4^+$ T cells recognize donor antigens. Depending on the type and levels of cytokines present after the engraftment, naïve $CD4^+$ helper T cells, considered the main actor of rejection, can acquire a different phenotype, differentiating into cytopathic or immune-regulatory immune cells. On the basis of current knowledge, the balance between rejection and tolerance mirrors the balance between cytopathic Th1 and Th17 $CD4^+$ T cells versus rejection-blocking, cytoprotective regulatory T cells [10]. Among the many factors known to modulate the immune pathophysiology of rejection, genetic polymorphisms of IL-6 [12], TNF-α [30], CTLA-4 [13] and IFN-γ [12] have recently attracted the attention of researchers.

In the last few years, a putative role in this field has emerged for vitamin D and its immune-modulator effects. Animal models have demonstrated that calcitriol administration prevented ACR and improved survival [31]. Immune-regulatory actions of vitamin D are thought to be exerted through the nuclear *VDR*, expressed in antigen-presenting cells and activated T cells [32,33]. *VDR*

determines interference and/or direct interaction with vitamin D responsive elements in the promoter regions of cytokine genes. Among the cells involved in the immune response, dendritic cells are primary targets for the immune activity of vitamin D; this occurs through the down-regulated expression of MHC class II, co-stimulatory molecules and IL-12 [34,35]. On the other hand, vitamin D enhances IL-10 production and promotes dendritic cells apoptosis. Altogether, these effects inhibit dendritic cell-dependent T-cell activation favouring the induction of regulatory, rather the cytopathic, T cells [34,35].

Vitamin D receptor gene is located in the long arm of chromosome 12 (12q12–14) and is composed of 10 exons, the first of which is not transcribed [16]. The polymorphic loci recognozed by BsmI G>A (rs1544410) and ApaI T>G (rs7975232) are located in intron 8, while the locus recognized by TaqI T>C (rs10735810) is located in exon 9. The last polymorphism leads to a silent codon change in which both alleles (ATT>ATC) code for isoleucine. The FokI C>T (rs10735810) polymorphic site is located three codons downstream the start site, thus creating an alternative initiation codon [25].

This study, for the first time, points out a strict relationship between recipient *VDR* polymorphisms and the occurrence of ACR in LT recipients. In particular, a significant association was detected between the carriage of the major allele of BsmI G>A and TaqI T>C polymorphic loci and occurrence of at least one ACR in the first year following LT. Among the three genetic models generally adopted, the recessive model for the major allele gave the major significance to the associations. Locus-by-locus AMOVA confirmed the major role played by the above-mentioned loci, while a minor association was detected for ApaI T>G. On the contrary, *VDR* FokI C>T polymorphism was not found to play a role. The only other study dealing with this topic in the literature found no association between the FokI C>T polymorphism of the recipient and ACR [36]. Our findings confirm this lack of an association with regard to this specific polymorphism, but indicate at the same time that other loci in the *VDR* gene are in fact strongly associated with ACR. However, it is important to outline that conflicting results obtained in different studies may be related, at least in part, to the ethnicity of the population involved. In fact, the presence of the A allele of BsmI G>A polymorphism was found to be associated with the occurrence of primary biliary cirrhosis in three studies involving Caucasian and Japanese populations [19,20,22], while in other two studies performed in Caucasian and Chinese populations, [18,21] primary biliary cirrhosis was found to be related to the presence of the G allele. Similarly, considering patients with autoimmune hepatitis, Vogel *et al.* [18] found an association of this disease with the C allele of the FokI C>T polymorphism, whereas opposite results were observed by Fan *et al.* [21]. Although a number of potential confounders may be advocated to explain these discrepancies, three major potential biases may be hypothesized. First, large differences exist in genotypic frequencies among different populations [16]. In fact, the frequency of the A allele of BsmI G>A polymorphism varies from 42% in Caucasian subjects to 36% in Africans and to 7% in Asians. Secondly, not all studies had sufficient sample size; this could provide a limited power to the results. Thirdly, only one or two polymorphisms were simultaneously determined, preventing a multivariate approach to be performed and a full comparison with other studies to be possible. This study, involving Italian liver-transplanted patients analysed for the four more common polymorphisms, might be considered for adequate homogeneity and statistical strength.

The genomic region encompassing the BsmI, ApaI and TaqI is located in the 3′ end of the *VDR* gene. This region, considered to be in linkage disequilibrium, as confirmed in the present study, is strictly linked to the poly(A) microsatellite repeat in 3′UTR genomic region known to be regulatory for mRNA stability [16]. Moreover, TaqI T>C polymorphic locus was found to be associated with the presence of CpG regulatory islands 5 and 6 [37]; through site-specific methylation or regional mechanisms, these regions are capable of modulating transcription factor binding and gene expression [38]. In experimental models, the carriage of BsmI A and TaqI C alleles could increase *VDR* expression, thus allowing an immune-regulatory effect [16]. The opposite effect may be expected when the G and T alleles are present in the *VDR* genome. Our results on diplotype analysis strongly support this assumption. Patients carrying the G-*-T/G-*-T diplotypes of the BsmI G>A, ApaI T>G and TaqI T>C polymorphic loci, experienced ACR earlier and more frequently than the remaining patients; the influence exerted by the carriage of these diplotypes was confirmed at multivariate analysis, either with stepwise logistic regression analysis or using the Cox proportional hazard model. Moreover, the observed differences in the rate of ACR occurrence in relationship with the carriage of a specific *VDR* diplotype would not depend on the intensity and the type of the immune-suppressive regimen adopted.

The study has some limitations. First, this is a retrospective analysis of a cohort of patients. Secondly and perhaps more importantly, only recipients' (not donors') genetic polymorphisms were available for study. It could be argued, however, that with regard to ACR occurrence in LT patients, recipient's rather than donor's immune response should be predominant [39]. Thirdly, caution should be used before extrapolating these results to other ethnicities, as allele frequencies of the *VDR* polymorphic region have large ethnic variability, as underlined above.

In conclusion, recipient *VDR* polymorphic loci in the 3′end region are strongly associated with occurrence of ACR during the first year after LT. The knowledge of *VDR* genetic polymorphisms may be helpful in identifying recipients at higher risk of ACR and in selecting them for a more aggressive immunosuppressive therapy.

Authorship

EF: designed the research. DB, AC, EF, SC, EF and GB: performed the research. EF, CA and EC: collected data. CF and EF: analysed the data. DB, CF, EF, MP and PT: wrote the manuscript.

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