

## ORIGINAL ARTICLE

# The presence of functional CCR5 and EBV reactivation after allogeneic haematopoietic stem cell transplantation

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EBV reactivation is a serious complication affecting the recipients of allogeneic haematopoietic stem cell transplants (allogeneic HSCT). Recent reports have suggested that EBV reactivation induces increased expression of C-C chemokine receptor-5 (CCR5) or its ligands. Therefore, the 32-nucleotide deletion within the CCR5-encoding gene (CCR5Δ32 polymorphism) was analysed in 92 recipients of allogeneic HSCT and their donors and related with EBV load. In addition in 30 patients, at the same time points employing a real-time PCR technique, the number of viral copies and CCR5 transcripts were assessed. The incidence of EBV reactivation 2–3 months after transplantation was significantly lower in patients carrying the CCR5Δ32 allele ( $P=0.008$ ). The association was confirmed in multivariate analysis, in which recipient CCR5Δ32 (OR = 0.166,  $P=0.026$ ) in addition to recipient age (OR = 1.536,  $P=0.034$ ) were identified as independent risk factors for EBV reactivation. Moreover, EBV reactivation was more frequently seen when patients and their donors were lacking the CCR5 deletion mutation as compared to other donor–recipient pairs ( $P=0.022$ ). The CCR5 expression was significantly higher in the group of patients having EBV reactivation than in those lacking it ( $R=25.354$ ,  $P=0.024$ ). These results suggest that the expression of functional CCR5 plays a role in initiation/perpetuation of EBV reactivation.

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## Introduction

Human C-C chemokine receptor 5 (CCR5) is a receptor for  $\beta$ -chemokines that are regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5) and also macrophage-inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$  (CCL3 and CCL4). Recent reports have suggested a potential role for CCR5 in perpetuation of viral infections, such as HIV (reviewed in Lusso<sup>1</sup>) or hepatitis C virus (HCV) infection.<sup>2</sup> CCR5 as a co-receptor for HIV-1 together with CXCR4 facilitate the fusion of HIV-1 with the plasma membrane of CD4+ cells.

Chemokines are critically involved in the process of perpetuation of viral infection, since they exert both chemotactic and immunoregulatory actions. In particular, the interaction between chemokines MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES (CCL3–5) and their receptor CCR5 may be critical in regulating T-cell functions by mediating recruitment, polarization, activation and differentiation of antiviral type 1 cytokines secreting T helper and cytotoxic T cells.

The human CCR5 gene maps to the short arm of chromosome 3p21.31. The 32-bp deletion mutation (CCR5Δ32) within the CCR5 gene results in a non-functional chemokine receptor. This mutation is common in Caucasoids, with a frequency of 13% in some Northern European populations, while absent in Africans and most Asian populations. The 32-bp deletion causes a shift in the reading frame, which results in a severely truncated protein that is unable to reach the cell surface. This leads to the complete loss of the functional CCR5 receptor in subjects homozygous for this mutation and decreased expression in heterozygous individuals.<sup>1</sup> It has also been documented that CD3-positive cells derived from CCR5Δ32 heterozygotes have a reduced surface expression of CCR5 and a weaker response to its ligands.<sup>3</sup> CCR5Δ32 does not produce a functional protein, explaining the near-complete protection against HIV-1 infection in individuals homozygous for the allele. Individuals heterozygous for the mutation express lower levels of CCR5 correlating with relatively low viral loads and slower progression to AIDS.<sup>1</sup>

EBV reactivation is a serious complication in the recipients of allogeneic haematopoietic stem cell transplantations (allogeneic HSCT). The recent reports have suggested that EBV reactivation induces increased

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expression of MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES (CCL3–5) and their receptor CCR5.<sup>4–6</sup> Therefore in the present study involving patients and donors of allogeneic HSCT, we wanted to determine if there is any relationship between the presence of the CCR5 deletion mutation and the risk EBV reactivation assessed by the number of EBV-DNA copies. In addition, CCR5 gene expression in relation to EBV load was studied.

## Patients, materials and methods

### Characteristic of the allogeneic HSCT patient group

Ninety-two patients (76 adults above 16 years of age and 16 children) who received allogeneic HSCT in our BMT Unit and 88 stem cell donors were studied for the CCR5 gene polymorphism in relation to transplant outcome. This work was approved by the Local Ethics Committee.

Fifty-two patients were transplanted from matched sibling donors while 35 and 5 patients were grafted from matched unrelated (MUD) or family haploidentical donors, respectively. For HLA-matched sibling transplants, HLA typing had been performed either serologically or by low-resolution molecular typing for HLA-A and -B and by high-resolution DNA typing for DRB1. Family haploidentical and unrelated patient–donor pairs were matched on the basis of DNA high-resolution typing for HLA-A, HLA-B, HLA-C, DRB1 and DQB1.

The patients group differed with respect to the conditioning regimen (myeloablative or reduced intensity conditioning), source of stem cells (bone marrow or peripheral blood stem cells), graft-versus-host disease (GvHD) prophylaxis (cyclosporine (CsA) or CsA with methotrexate or CsA with mycophenolate mofetil) and donor–recipient gender relation (sex matched or mismatched); see Table 1.

Acute GvHD was graded according to the consensus conference on acute GvHD grading.<sup>7</sup> Among 92 patients, 55 did not develop acute GvHD, whereas 20 had grades II–IV GvHD (Table 1). Thirty-seven patients were presented with chronic GvHD. Sixteen patients died after transplantation (among them two before +100 days post transplant).

### CCR5 genotyping

DNA was extracted from EDTA peripheral blood using silica membranes (QiAmp Blood kit and RNeasy Mini kit, Qiagen, Hilden, Germany) following the recommendation of the manufacturer. The 32-nucleotide deletion within the CCR5 encoding gene (CCR5 $\Delta$ 32 polymorphism) was analysed by a PCR technique as described previously,<sup>8</sup> with some modifications. In brief, a pair of primers (5'-CTTCATTACACCTGCAGCTCT-3'; 5'-CACAGCCC TGTGCCTCTTCTTC-3') flanking the region of 32-nucleotide deletion in the CCR5 gene was used to generate wild-type and deleted DNA fragments of 182 and 150 bp, respectively. PCR amplification was conducted at 94°C, 3 min, followed by two cycles of (94°C, 40 s; 68°C, 40 s; 72°C, 40 s) then two cycles with an annealing temperature of 64°C and 62°C and 30 cycles of (94°C, 40 s; 58°C, 40 s; 72°C, 40 s) with a final elongation step 72°C, 5 min.

**Table 1** Patients' characteristics

Number of patients	92
Age (median, range) years	0.3–60; 28.5
Adults > 16 years	76
Children $\leq$ 16 years	16
Donor–recipient gender	
Female–male	32
Other combinations	60
Donor	
Sibling	52
Alternative (family haploidentical/matched unrelated)	40 (5/35)
Transplant material	
BM	27
PBPC	65
Diagnosis	
Haematological malignancies (HM)	77
CML	32
AML	19
ALL	11
Other HM	35
Anaemias and immunodeficiencies	15
Conditioning regimen	
Myeloablative <sup>a</sup>	40
Reduced intensity conditioning (RIC) <sup>b</sup>	52
GvHD prophylaxis	
CsA	72
CsA + (MTX or MMF)	20
Acute GvHD, grades	
0	55
I	17
II	13
III	4
IV	3
Chronic GvHD	37
EBV copies < 10/10 <sup>5</sup> cells	56
EBV copies $\geq$ 10/10 <sup>5</sup> cells	36
Recipients carrying the CCR5 $\Delta$ 32 mutation	
Homo-/heterozygotes	2/15
Donors carrying the CCR5 $\Delta$ 32 mutation	
Homo-/heterozygotes	0/9

<sup>a</sup>Standard myeloablative: Bu (16 mg/kg b.w.) + Cy, aggressive myeloablative: Bu (16 mg/kg b.w.) + Cy + (Vp or TT or ATG).

<sup>b</sup>RIC: Bu (8 mg/kg b.w.) or Mel (140 mg/m<sup>2</sup>) + Flu + ATG.

### Real-time TagMan assay for EBV quantification

EBV load was analysed in healthy individuals, and in patients before and 2–3 months after transplantation (1–7 analyses, with two measurements per patients in an average 2–3 months post-transplant). In the latter period, the peak value of EBV-DNA copies was considered in further analyses if more than one measurement was done for a given patient.

Similar time points (2 and 3 months after transplantation) were identified by Wang *et al.*<sup>9</sup> and Hoshino *et al.*,<sup>10</sup> who analysed the kinetics of detection of EBV DNA before and after HSCT, as characterized with either the higher

frequencies of patients positive for EBV DNA in peripheral blood or by the higher numbers of EBV viral copies per  $\mu\text{g}$  of DNA.

The evaluation of the EBV-specific DNA copies was performed using TaqMan chemistry with detection on Opticon 2 (MJ Research, Waltham, MA, USA) as described previously.<sup>11</sup>

Considering that virus-infected cells constitute approximately 1 in  $10^4$ – $10^5$  memory B lymphocytes,<sup>12</sup> we assumed the detection threshold for viral reactivation of our method as 10 EBV-DNA copies/ $10^5$  peripheral blood cells.

The quantitation of EBV DNA performed in an unrelated healthy population ( $n = 74$ , F/M: 51/23, aged 21–55 years, median 25 year) showed that all these individuals presented with less than 10 EBV-DNA copies/ $10^5$  cells (0.0–6.1 EBV-DNA copies/ $10^5$  cells; average 0.54 copy/ $10^5$  cells), in agreement with the definition of viral reactivation used in the present study.

#### CCR5 gene expression

The quantification of the CCR5 transcripts was performed in 31 patients for whom RNA samples from the appropriate time post-transplant were available. CCR5 gene expression was analysed by a real-time PCR technique using SYBR-Green I technology<sup>13</sup> and calculated according to Pfaffl,<sup>14</sup> using a relative expression software tool (REST)<sup>15</sup> and given as expression ratio (ER) coefficient  $R$ . Calculations were performed in relation to the expression of  $\beta$ -actin gene.

#### Statistical analysis

Univariate analyses of the distribution of CCR5 genotypes in patients having and lacking EBV reactivation were performed by the Fisher exact test or  $\chi^2$ -test as appropriate. The Statistical Package for Social Scientists (SPSS, SYSTAT 10) was used for complete multivariate logistic regression analysis.

The PairWise Fixed Reallocation Radomization Test<sup>14,15</sup> was employed to compare the CCR5 expression in the group of patients having and lacking EBV reactivation.

Probability values  $<0.05$  were considered statistically significant, and those between 0.05 and 0.1 as indicative of a trend.

## Results

#### Comparison of patients having and lacking EBV reactivation

The patients were divided into groups having  $<10$  EBV-DNA copies and  $\geq 10$  EBV-DNA copies/ $10^5$  peripheral blood cells and were stratified with respect to the other factors known to be associated with the risk of post-transplant complications.<sup>16</sup> The details are given in Table 2. The only significant differences were associated with the CCR5 gene polymorphism.

Among others, no significant relationship was observed between the donor and/or recipient serological status before transplantation. No association was also seen when the numbers of EBV-DNA copies in donor (0.0–51 copies/

$10^5$  cells, average 3.4 copy/ $10^5$  cells) and recipient (0.0–290 copies/ $10^5$  cells, average = 7.4 copy/ $10^5$  cells) samples taken before transplantation were considered. There were only seven cases (two patients and five donors) with more than 10 EBV-DNA copies/ $10^5$  cells. None of the patients who had more than 10 EBV-DNA copies/ $10^5$  cells before transplantation presented with elevated numbers of EBV copies 2–3 months post-transplant. Increased numbers of EBV copies after transplantation were also not detected in any of the patients grafted from donors having greater than 10 EBV-DNA copies/ $10^5$  cells.

No relationship was found between the presence of GvHD symptoms or CMV reactivation, assessed by the presence of IgM and/or CMV-DNA copies (Table 2).

#### Relationships between the presence of the CCR5 deletion mutation and EBV load

A lower incidence of EBV reactivation 2–3 months after transplantation was observed in recipients with the CCR5 deletion mutation. For this period, the peak value of EBV-DNA copies was considered in further analyses if more than one measurement was carried out for a given patient.

The higher numbers of EBV-DNA copies were detected in patients lacking the CCR5 $\Delta 32$  allele. Among 75 patients lacking the CCR5 deletion mutation, 34 presented with EBV infection as compared with 2 out of 17 patients carrying the CCR5 $\Delta 32$  allele (0.45 vs 0.12,  $P = 0.008$ , Figure 1a, Table 2).

No direct significant association was observed for donor genotype and EBV reactivation. However, this complication was more frequently seen when patients and their donors were lacking the CCR5 deletion mutation as compared to the other donor–recipient pairs (0.46 vs 0.19,  $P = 0.022$ , Table 2).

Association of recipient CCR5 genotype was also valid after adjusting for the other variables (recipient and donor age and sex, donor–recipient gender relation, type of donor, donor–recipient viral serological status before transplantation, conditioning regimen, diagnosis, GvHD, source of HSC) in multivariate logistic regression analysis (SPSS, SYSTAT10). It appeared that two variables significantly associated with the risk of EBV reactivation: recipient age (more than 25 years old) (OR = 1.536, 95% CI: 1.136–2.703,  $P = 0.034$ ) and recipient CCR5 $\Delta 32$  allele (OR = 0.166, 95% CI: 0.034–0.803,  $P = 0.026$ ).

#### Association between the CCR5 gene expression and EBV load

To study the relationship between CCR5 gene expression and EBV reactivation, the viral load and number of CCR5 gene transcripts were assessed at the same time points 2–3 months following transplantation. CCR5 gene expression was analysed by a real-time PCR technique using SYBR-Green I technology<sup>13</sup> and calculated according to Pfaffl,<sup>14</sup> using a REST<sup>15</sup> and given as ER coefficient  $R$ . Calculations were performed in relation to the expression of  $\beta$ -actin gene. A greater EBV load ( $\geq 10$  copies/ $10^5$  cells) was seen in patients with enhanced CCR5 gene expression. It appeared that the CCR5 expression in the group of patients having EBV reactivation was upregulated (by the factor  $R = 25.354$ )

**Table 2** Comparison of patients having and lacking EBV reactivation

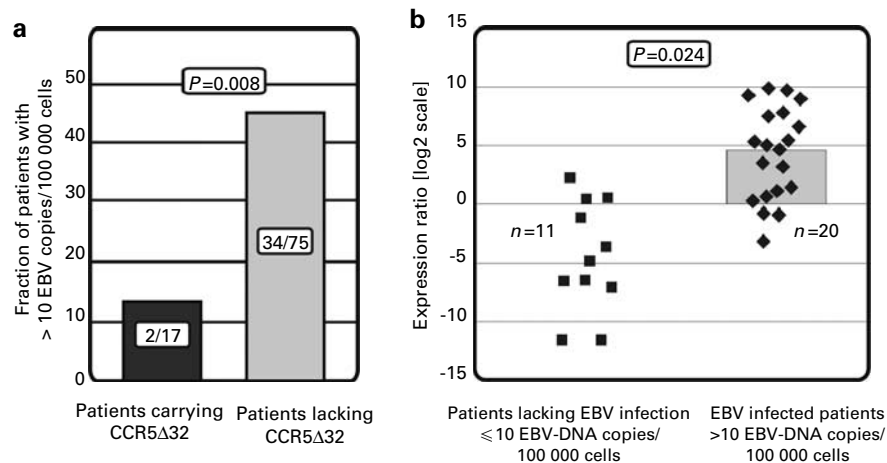
Factor	Cases with a given factor among patients (%)		P
	Without EBV reactivation	With EBV reactivation	
Recipient CCR5 mutation	27	6	<b>0.008</b>
Donor CCR5 mutation	13	6	0.223
Recipient and donor CCR5 mutation	6	0	0.214
Recipient or donor CCR5 mutation	32	11	<b>0.022</b>
Alternative donor	46	43	0.408
<i>Recipient</i>			
> 16 years of age	82	86	0.559
> 25 years of age	48	63	0.159
> 35 years of age	43	43	0.542
Female to male transplantation	38	31	0.360
Haematological malignancies	86	83	0.354
Myeloablative conditioning regimen	52	34	<b>0.063</b>
GvHD prophylaxis with CsA monotherapy	88	78	0.206
aGvHD (I–IV)	38	46	0.327
aGvHD (II–IV)	18	31	0.123
aGvHD (III–IV)	5	11	0.266
CGvHD	42	41	0.521
BM as a source of HSC	29	29	0.564
Fatal cases	16	20	0.564
Use of ATG	63	54	0.239
EBV donor serology positive	82	92	0.251
EBV recipient serology negative	14	17	0.494
EBV serology donor positive/recipient negative	6	10	0.428
CMV reactivation	39	40	0.185

Significant differences ( $P < 0.05$ ) and strong tendencies ( $0.05 < P < 0.1$ ) between two groups of patients are given in bold.

EBV reactivation was defined as the presence of  $\geq 10$  EBV-DNA copies/ $10^5$  peripheral blood cells.

EBV donor and/or recipient serology represents donor or recipient serological status before transplantation.

CMV reactivation was assessed by the presence of CMV-DNA copies or IgM after transplantation.



**Figure 1** Relationship of CCR5 gene polymorphism and the number of CCR5 transcripts with EBV load. (a) A lower incidence of EBV reactivation 2–3 months after transplantation was observed in recipients with the CCR5 deletion mutation. For this period, the peak value of EBV-DNA copies was considered in further analyses if more than one measurement was carried out for a given patient. (b) Viral load and the numbers of mRNA CCR5 copies were assessed at the same time points 2–3 months after transplantation employing a real-time PCR technique. Patients with increased CCR5 expression more frequently presented with the elevation of EBV copies ( $> 10$  copies/ $10^5$  cells). The bar in the figure represents the median value of the CCR5 expression ratio in patients with EBV reactivation. Note that, probably due to the small number of patients carrying the CCR5 $\Delta 32$  allele analysed for CCR5 gene expression, no association was observed between the expression and polymorphism of the CCR5 gene. Among patients for whom the CCR5 gene expression was analysed, there were only two patients carrying the CCR5 deletion mutation (both homozygous for the CCR5 $\Delta 32$  allele) in the group lacking EBV infection and two heterozygous patients among those with EBV infection. CCR5, C-C chemokine receptor-5; ER, expression ratio.

and significantly different from the CCR5 expression in the group of patients lacking EBV reactivation ( $P = 0.024$  PairWise Fixed Reallocation Radomization Test,<sup>14,15</sup> Figure 1b).

Among patients in whom the CCR5 gene expression was analysed, there were only two patients carrying the CCR5 deletion mutation (both homozygous for the CCR5 $\Delta 32$  allele) in the group lacking EBV reactivation and two

heterozygous patients among those with EBV reactivation. Both patients homozygous for the CCR5 $\Delta$ 32 allele in the group lacking EBV reactivation had decreased CCR5 expression (ER <0, Figure 1b) while in two other patients carrying the CCR5 $\Delta$ 32 allele, among those presented with EBV reactivation, CCR5 gene expression was increased (ER >0, Figure 1b).

## Discussion

In the present study, we found two previously undescribed associations of the presence of CCR5 $\Delta$ 32 allele or decreased CCR5 gene expression with the lower EBV load in patients after transplantation. The association of CCR5 polymorphism with the risk of EBV reactivation was confirmed in multivariate analysis. In this analysis, the presence of CCR5 $\Delta$ 32 allele in the recipient was identified as an independent risk factor for EBV reactivation in addition to recipient age. These results imply that the presence of the functional CCR5 receptor (assessed either by the lack of the 32-bp deletion within the CCR5 gene or its higher expression) plays a role not only in AIDS but may also be involved in reactivation/infection of other viruses, as shown in the present study for EBV.

Interestingly, it has been reported that EBV-encoded LMP-1 induces expression of CCL5 mRNA and secretion of CCL5,<sup>4</sup> which may result in increased expression of CCR5. EBV-transformed B cells also secrete CCL3 and CCL4, two further ligands for CCR5.<sup>6</sup> Furthermore, it has been shown that in acute EBV infection, proliferating CCR5+CD4+ T cells are accumulated to very high levels.<sup>5</sup> Therefore, the observed increase in the number of CCR5 transcripts in patients presented with the increased EBV load, initiated by EBV replication within B cells, may affect the functions of activated T lymphocytes and as a result could induce the better response of T cells within the site of inflammation/infection.

By contrast, it has been shown in murine models of acute GvHD that CCR5-expressing T lymphocytes are recruited to the liver during acute GvHD in mice while disrupting the gene encoding CCR5 interrupts recruitment of T cells into gut Peyer's patches and as a result prevents acute GvHD.<sup>17</sup> Our previous study has documented that the presence of the 32-bp deletion within the CCR5 gene associates with a lower risk of acute GvHD after allogeneic HSCT in humans,<sup>18</sup> suggesting that the deletion within the chemokine receptor can affect cell trafficking by decreased interaction with its ligands.<sup>19</sup> Taking into account that viral reactivations may promote GvHD,<sup>20</sup> it could also be assumed that the expression of functional CCR5 may influence the initiation/perpetuation of GvHD by promotion of viral reactivation.

In fact, in this present study, no significant relationship was found between the presence of GvHD symptoms and the risk of EBV reactivation in either uni- or multivariate analysis. Only a slight prevalence of acute GvHD grades II–IV was observed among patients with >10 EBV-DNA copies/10<sup>5</sup> blood cells as compared to those without EBV reactivation (0.52 vs 0.35,  $P=0.123$ ). Thus, the previously reported relationship between the CCR5 $\Delta$ 32 allele and

acute GvHD could be due to less effective antiviral surveillance directly associated with the CCR5 deletion mutation. However, this hypothesis warrants further study.

The present study documents the previously not described associations between polymorphism and expression of the CCR5 gene and EBV load. These data support the theory that a patient's CCR5 genotype may identify individuals less or more prone to EBV reactivation. They might also allow identification of patients at high risk or in the early stage of EBV reactivation after transplantation. This may enable prophylactic treatment or an attempt to cure the infection by administering anti-CD20 monoclonal antibody or EBV-CTL, or reducing the immunosuppressive therapy. Thus, CCR5 and probably also its ligands might appear to be the novel target molecules of therapeutic intervention in patients undergoing allogeneic HSCT.

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