



CTLA-4 +49A>G polymorphism of recipients of HLA-matched sibling allogeneic stem cell transplantation is associated with survival and relapse incidence

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Abstract: Conflicting observations have been reported about the role of CTLA-4 gene polymorphisms in the clinical outcome of allogeneic hematopoietic stem cell transplantation (HSCT). We have investigated 3 polymorphisms of the CTLA-4 gene (-318C>T, +49A>G, CT60G>A) in 133 donor/recipient pairs who underwent HLA-matched sibling donor HSCT for hematological malignancies. We found no association of the clinical outcome of the HSCT with either recipient or donor -318C>T and CT60G>A polymorphisms. At variance, we found a significant association of donor +49A>G GG genotype with longer overall survival (OS) (log rank P=0.04), and the number of +49A>G G-alleles in the recipient with longer OS (P for trend=0.027), longer disease-free survival (P for trend=0.036) and reduced relapse rate (P for trend=0.042). However, only recipient +49A>G polymorphism was retained as independent prognostic factor in a multivariate analysis, suggesting that the expression of CTLA-4 on the cells of recipient may be relevant for the clinical outcome of HSCT.

Response to Reviewers: Jurgen Finke, MD
Associate Editor
Annals of Hematology

December 2 , 2009

Dear Editor,

I am hereby submitting the third revised version of our manuscript Ref. No: AOHE-D-09-00069 entitled "CTLA-4 +49 A>G polymorphism of recipients of HLA-matched sibling allogeneic stem cell transplantation is associated with survival and relapse incidence".

As suggested, we have now addressed the minor comments of Reviewer #1 as follows. Modifications are underlined.

REVIEWER #1

Point 1

According to the Reviewer's request, we have indicated in the figure that the p values are from the log rank test. In the figure legend, we have also specified that the log rank test estimates differences in terms of OS or DFS among the 3 genotypes of Recipient considered as separate discrete categories (+49GG, AG, AA).

As requested we have included "*log rank p=0.027" and "*log rank p=0.036" at the bottom left of the OS and DFS graphics, respectively, in Figure 1 and the following new sentence: "* the P values, obtained by log rank tests, refer to the differences in terms of OS and DFS across the 3 subgroupss of the discrete variable (recipient genotype)" in the Legend to Figure 1.

Point 2

In the last paragraph of Results, page 8, line 1, the word "response" has been deleted with the new sentence becoming: "In view of these results and of the known unfavorable effect of acute GvHD on both OS and DFS, we further investigated a possible correlation between acute, as well as chronic GvHD, and the +49A/G or G/G recipient genotype".

Point 3

Since this statistical analysis was not performed, we agree with the Reviewer that our sentence was too speculative. We thus have deleted the following sentence: "According to this hypothesis, the GvL effect in our series of patients was independent from the severity of GVHD" in the Discussion Page 10, lines 8-9.

We believe that we have addressed the minor concerns raised by the Reviewer #1 and hope that the revised manuscript will meet the requirements for publication in Annals of Hematology.

We thank you very much for your attention and look forward to hearing from you.

Yours sincerely,

Maria Pia Pistillo

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CTLA-4 +49A>G polymorphism of recipients of HLA-matched sibling allogeneic stem cell transplantation is associated with survival and relapse incidence.

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Brief title: CTLA-4 +49 A>G polymorphism and HSCT outcome

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Abstract

Conflicting observations have been reported about the role of *CTLA-4* gene polymorphisms in the clinical outcome of allogeneic hematopoietic stem cell transplantation (HSCT). We have investigated 3 polymorphisms of the *CTLA-4* gene (-318C>T, +49A>G, CT60G>A) in 133 donor/recipient pairs who underwent HLA-matched sibling donor HSCT for hematological malignancies. We found no association of the clinical outcome of the HSCT with either recipient or donor -318C>T and CT60G>A polymorphisms. At variance, we found a significant association of donor +49A>G G/G genotype with longer overall survival (OS) (log rank $P=0.04$), and the number of +49A>G G-alleles in the recipient with longer OS ($P=0.027$), longer disease-free survival ($P=0.036$) and reduced relapse rate ($P=0.042$). However, only recipient +49A>G polymorphism was retained as independent prognostic factor in a multivariate analysis, suggesting that the expression of *CTLA-4* on the cells of recipient may be relevant for the clinical outcome of HSCT.

Keywords: CTLA-4 polymorphism; transplant outcome; survival; disease relapse

Introduction

Cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) is a negative regulator of T-cell activation [1,2] which, as suggested by studies in animal models of transplant [3-5], may contribute to modulate immune response and tolerance in both solid organ and hematopoietic stem cell transplantation (HSCT).

Few polymorphisms in the *CTLA-4* gene, likely because they may affect the inhibitory function of CTLA-4 [6,7], have been found associated with the susceptibility to autoimmune diseases [6-9] and recently with the outcome of allogeneic hematopoietic stem cell transplantation (HSCT). In a multicentric study, Perez-Garcia et al. have investigated *CTLA-4* gene polymorphisms in human leukocyte antigen (HLA)-identical sibling donor HSCT; they found that the presence of at least one G-allele of CT60 polymorphism in the donors was associated with reduced overall survival (OS), higher relapse risk and lower risk of acute graft-versus-host disease (GvHD) [10]. Vannucchi et al., in unrelated HLA-matched donor HSCT, confirmed the association with GvHD but not with OS and relapse [11]. Azarian et al., in HLA-identical sibling donor HSCT, found the association of chronic GvHD only with donor +49A>G G/G genotype [12].

In this study, we have retrospectively investigated the influence of both donor and recipient *CTLA-4* gene polymorphisms (-318 C>T, +49 A>G and CT60 G>A) on the clinical outcome of HLA-identical sibling donor HSCT.

Material and Methods

Patients The study included 133 consecutive donor-recipient pairs in which the recipient underwent HLA-identical sibling donor HSCT for hematological malignancies in a single center (San Martino Hospital, Genoa, Italy) between 1994 and 1999. All patients underwent a long follow-up observation (median 6.5 years, range 13 days-12.2 years). The informed consent was obtained according to institutional procedures and to the declaration of Helsinki. Patient characteristics are summarized in Table 1. The median age of patients was 40 years (range 17-65) and that of donors was 38 years (range 10-59). Sex mismatch between donor and recipient was present in 71 pairs. All patients have been transplanted with unmanipulated HLA-identical sibling hematopoietic stem cells derived either from peripheral blood ($n= 95$) or from bone marrow ($n= 38$). One hundred and twenty-six patients received a myeloablative conditioning regimen that in 81 patients included the total body irradiation (TBI), and 7 patients received a reduced intensity conditioning regimen. The prophylaxis of GvHD was performed in all patients with cyclosporine and methotrexate (MTX). Twenty-seven patients received donor lymphocyte infusions (DLI).

CTLA-4 genotyping

Whole peripheral blood samples were collected from donors and from recipients before HSCT and genomic DNA was extracted by standard methods. *CTLA-4* -318 C>T (promoter) and +49 A>G (exon 1) polymorphisms have been genotyped by a previously described tetra-primer amplification refractory mutation system (T-ARMS-PCR) [13,14]. CT60 G>A (3' UTR) polymorphism has been genotyped by a newly developed T-ARMS-PCR (details available on request).

Statistical analysis

Deviation from Hardy Weinberg equilibrium (HWE) was tested with Pearson's χ^2 test using the Finetti program (Authors: Wienker & Strom; <http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>).

Overall survival (OS) and disease-free survival (DFS) have been analyzed by Kaplan Meier method and compared using the log-rank test. Multivariate Cox regression models (backward stepwise method) have been performed to evaluate the combined effect of the following variables: age and sex of recipient and of donor, sex mismatch, type and stage of disease, source of stem cells, TBI, and donor and recipient +49A>G genotypes. Since investigated polymorphisms are likely to affect gene expression (or the activity of the gene product), it is expected that the levels of expression in heterozygotes are intermediate in the respect of homozygotes: thus, the association with genotypes have been tested with Mantel-Haenszel chi-square test for trend. Kaplan-Meier analyses and Cox's regressions were performed using the SPSS package (version 13.0 for Windows). All statistical tests were two-sided; statistical significance has been accepted for any $P < 0.05$.

Results

We have genotyped 3 polymorphisms of *CTLA-4* gene (-318C>T, promoter; +49A>G, exon 1; CT60G>A, 3'UTR) in 133 pairs of donors and recipients who underwent HLA-matched sibling donor HSCT for hematological malignancies in a single center. Genotype frequencies of all 3 polymorphisms (Table 2) fit the Hardy-Weinberg *equilibrium* in both recipients and donors and were similar to that found in the Italian population [13,15,16] and in the HSCT series reported by Perez-Garcia *et al.* [10].

We found no correlation between the +49A>G donor and recipient genotype and either one of acute or chronic GvHD. Similarly, none of the donor and recipient -318C>T or CT60G>A polymorphisms appeared associated with GvHD. Furthermore, transplant outcome (survival or disease relapse) was not associated with -318C>T or CT60G>A polymorphisms in either recipient or donor (not shown). In contrast with previous report [10], we have observed a longer OS in patients transplanted from a donor that was homozygote G/G for the +49A>G polymorphism (5-year OS: A/A, 51%; A/G, 46%; G/G, 87%; log rank $P=0.04$). The analysis of CTLA-4 polymorphisms in HSCT recipients, analysis that has not been performed in the previous studies [10, 12], has shown that the number of +49A>G G-alleles in the recipient was significantly associated with longer OS (5-year OS: A/A, 46%; A/G, 55%; G/G, 90%; log rank $P=0.027$; figure 1A), longer DFS (5-year DFS: A/A, 59%; A/G, 68%; G/G, 80%; log rank $P=0.036$; figure 1B) and reduced relapse rate (A/A, 43%; A/G, 28%; G/G, 20%; log rank $P=0.042$; data not shown). Interestingly, the total number of +49A>G G-alleles carried by each donor/recipient pair appeared associated with longer OS (5-year OS: 3 or 4-G-alleles, 87%; ≤ 2 G-alleles, 49%; log rank $P=0.02$); however, this association is mostly due to the sibling relationship between recipients and donors that results in a high proportion of pairs in which recipient and donor have the same genotype ($\chi^2=75.8$; $P<10^{-6}$). The relative role of donor and of recipient +49A>G polymorphism in the clinical outcome of HSCT has been

1 further evaluated by a multivariate Cox's regression model including relevant clinical
2 variables (listed in “material and methods”) and donor and recipient +49A>G polymorphism
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4 (Table 3). Only disease stage (advanced vs early) and the recipient +49A>G genotype have
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6 been retained in the final model of OS as significant independent predictors of survival; the
7
8 hazard of death was much higher in patients with advanced disease as compared to those with
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10 early disease (HR=2.63, 95%CI 1.59-4.34, $p<0.001$); with respect to the wild-type genotype
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12 A/A , the hazard of death was gradually decreasing according to the number of G-alleles in
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14 the recipient (HR for A/G genotype =0.67, 95%CI 0.40-1.10; HR for G/G genotype =0.09,
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16 95%CI 0.13-0.72, P for trend=0.014). Disease stage (HR=2.15 95%CI 1.19-3.89, $P<0.011$)
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18 and recipient +49A>G genotype (HR for A/G genotype =0.53, 95%CI 0.29-0.97; HR for G/G
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20 genotype =0.26, 95%CI 0.06-1.12, P for trend=0.025) were also significantly associated with
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22 DFS, as well as TBI (HR of absence of TBI treatment vs TBI treatment =2.84, 95%CI 1.55-
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24 5.24, $p<0.001$). Thus the multivariate analysis confirmed the independent prognostic
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26 significance of recipient, but not of donor, +49A>G genotypes for both OS and DFS.
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31 In view of these results and of the known unfavorable effect of acute GvHD on both OS and
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33 DFS, we further investigated a possible correlation between acute, as well as chronic GvHD,
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35 and the +49A/G or G/G recipient genotype. When considering these genotypes versus the
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37 wild type A/A genotype, we did not find any significant reduced severity of either one of the
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39 GvHD types (acute GvHD, OR of grade 0-1 vs 2-4: 1.02, 95%CI 0.51-2.03, $p=0.954$; chronic
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41 GvHD, OR of limited vs moderate-extenxsive: 1.84, 95%CI 0.86-3.85, $p=0.103$).
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Discussion

We have investigated CTLA-4 gene polymorphisms in a series of 133 HSCT recipients (and their HLA-matched sibling donors) that have been enrolled in a relatively short lapse of time (1994-99) in a single Center and that have a long follow-up (median 6.5 years). In this study, we have not confirmed the previously reported effects of donor +49A>G and of donor CT60G>A polymorphisms on the clinical outcome of HSCT [10]: in particular we have observed that patients transplanted from a donor carrying at least one G-allele of CT60 polymorphism have, without reaching statistical significance, a longer and not a reduced OS (5-year OS: A/A, 46%; A/G and G/G, 55%; $P=0.4$). Such discrepancies could be explained, in part, by the different clinical features of patients included in these series of HLA-matched sibling donor HSCT: in fact Perez-Garcia's study [10] included a smaller proportion of (i) gender mismatch (25% vs. 53%; $P<0.001$), (ii) bone marrow as stem-cells source (50% vs. 29%; $P<0.001$) and (iii) total body irradiation (38% vs. 61%; $P=0.001$).

On the other hand our data, supported by the multivariate analysis, show that recipient *CTLA-4* +49A>G gene polymorphism, which had not been investigated so far in the setting of HLA-matched sibling donor HSCT [10,12] may be relevant for the clinical outcome of HSCT. This surprising and unexpected finding suggests that *CTLA-4* might play a role in post-transplant control of disease through its expression on the recipient cells that include leukemic cells [17,18], recipient monocytes [19] and regulatory T cells of recipient origin that can persist after myeloablative treatments [20]. The +49A>G G-allele has been associated with the decreased expression of *CTLA-4* gene [21] and the overall reduced function of its product [6,7]: this could result in an increased activation of T cells that explains the reported association of +49A>G G-allele with the increased susceptibility to autoimmune disorders [6-9]. This is in keeping with the longer survival and the reduction of relapse rate we have

1 observed in allo-HSCT recipients bearing the +49A>G G-allele. These recipients may express
2 lower levels of *CTLA-4* on either leukemic (or micro-environment) cells, and this could result
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4 in a reduced interaction of *CTLA-4* with B7 molecules expressed on donor T cells. As it has
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6 been found that T cells may express B7 molecules able to transduce inhibitory signals [22],
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8 the reduced *CTLA-4:B7* interaction might represent a mechanism leading, eventually, to a
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10 more effective control of leukemic cells (graft-versus-leukemia, GvL) by the donor T cells.
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16 It is also suggestive that a recipient-derived GVL effect, in the context of an autoimmune
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18 disorder, has been observed in a murine model of hematopoietic transplant after the late
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20 blockade of *CTLA-4* [5]. In addition, in keeping with our finding, it has been recently reported
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22 that the blockade of the *CTLA4* molecule may be effective in the treatment of malignancy
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24 relapse after allo-HSCT [23]. For all these reasons, if confirmed, our data will provide
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26 additional information on the risk for allogeneic transplantation outcome.
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31 Although the intriguing biological hints of our findings, the data about *CTLA-4* gene
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33 polymorphisms and HSCT reported in various studies [10-12 and present report] remain
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35 highly conflicting. This suggests caution in evaluating the possible impact of *CTLA-4* gene
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37 polymorphisms on the clinical outcome of allogeneic HSCT; therefore, they require validation
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40 in properly designed prospective clinical trials.
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Legend to Figure 1

Kaplan-Meier survival curves according to the genotype of the CTLA-4 +49A>G gene polymorphism of the recipient (A/A, A/G and G/G).

A) Overall survival (OS). n = number of patients at risk. OS with 95% confidence interval (CI) in parentheses. $P=0.027$.

B) Disease free survival (DFS). n = number of patients at risk. DFS, with 95% CI in parentheses. $P=0.036$.

* the P values, obtained by log rank tests, refer to the differences in terms of OS and DFS across the 3 subgroupss of the discrete variable (recipient genotype).

TABLE 1. Patient characteristics

Feature	n (%)
Number of patients	133
Median age (range), y	40 (17-65)
Sex (male:female)	87:46
Diagnosis	
Acute lymphoblastic leukaemia, <i>n</i> (%)	17 (12.8)
Acute myeloid leukemia, <i>n</i> (%)	39 (29.3)
Myelodysplastic syndrome, <i>n</i> (%)	11 (8.3)
Chronic myeloid leukemia, <i>n</i> (%)	44 (33.1)
Chronic idiopathic myelofibrosis, <i>n</i> (%)	3 (2.2)
Lymphoproliferative disorders, <i>n</i> (%)	14 (10.5)
Hodgkin disease, <i>n</i> (%)	5 (3.8)
Disease stage (early:advanced)	80:53
Sex mismatch, <i>n</i> (%)	71 (53.4)
Source of stem cells	
Bone marrow, <i>n</i> (%)	38 (28.6)
Peripheral blood, <i>n</i> (%)	95 (71.4)
Conditioning regimens	
Myeloablative regimen with TBI, <i>n</i> (%)	81 (60.9)
Myeloablative regimen w/o TBI, <i>n</i> (%)	45 (33.8)
Reduced intensity conditioning regimen, <i>n</i> (%)	7 (5.3)
Acute GvHD \geq grade II, <i>n</i> (%)	58 (43.6)
Chronic GvHD \geq grade II, <i>n</i> (%)	52 (39.1)
Donor lymphocyte infusion, <i>n</i> (%)	27 (20.3)
<i>Abbreviations:</i> GvHD: graft-versus-host disease	

TABLE 2. Genotype and allele frequencies of CTLA-4 polymorphisms in recipients and donors of HLA-identical sibling HSCT

Genotypes	n (%)		Alleles	n (frequency)	
	Recipients	Donors		Recipients	Donors
-318 C>T	(n= 133)	(n= 133)	-318 C>T	(2n= 266)	(2n= 266)
C/C	106 (79.7)	111 (83.5)	C	237 (0.89)	242 (0.91)
C/T	25 (18.8)	20 (15.0)	T	29 (0.11)	24 (0.09)
T/T	2 (1.50)	2 (1.5)			
+49 A>G	(n= 133)	(n= 133)	+49 A>G	(2n= 266)	(2n= 266)
A/A	65 (48.9)	62 (46.6)	A	188 (0.71)	180 (0.68)
A/G	58 (43.6)	56 (42.1)	G	78 (0.29)	86 (0.32)
G/G	10 (7.5)	15 (11.3)			
CT60 G>A	(n= 133)	(n= 133)	CT60 G>A	(2n= 266)	(2n= 266)
G/G	35 (26.3)	32 (24.1)	G	125 (0.47)	128 (0.48)
A/G	55 (41.4)	64 (48.1)	A	141 (0.53)	138 (0.52)
A/A	43 (32.3)	37 (27.8)			

Abbreviation: HSCT, hematopoietic stem cell transplantation.

Figure 1

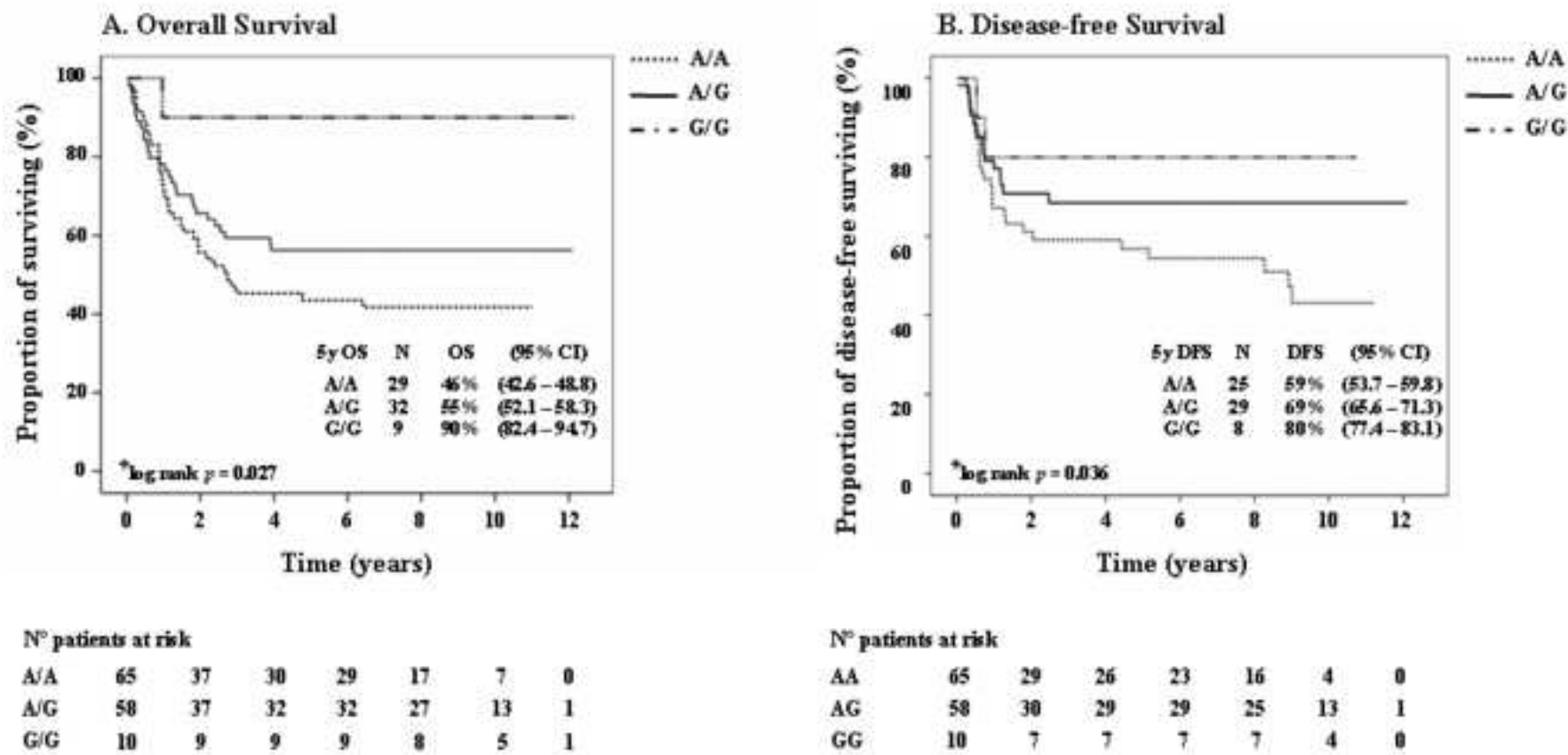


TABLE 3. Multivariate analysis : survivals according to prognostic factors.

Factor	Overall Survival			Disease-free Survival		
	HR [¶]	95% CI [#]	<i>p</i> value [‡]	HR [¶]	95% CI [#]	<i>p</i> value [‡]
Recipient age			0.734 ^{§*}			0.144 ^{§*}
≤25	1 (ref)	-		1 (ref)	-	
26-35 y	0.92	0.35-2.44		0.45	0.18-1.16	
36-45 y	1.12	0.37-3.36		0.48	0.20-1.17	
>45 y	0.89	0.25-3.15		0.70	0.31-1.57	
Donor age			0.419 ^{§*}			0.219 ^{§*}
≤30 y	1 (ref)	-		1 (ref)	-	
31-40 y	1.49	0.72-3.09		2.08	0.66-6.54	
41-49 y	1.20	0.56-2.59		2.86	0.72-3.42	
>49 y	1.53	0.69-3.37		3.09	0.62-5.29	
Recipient gender			0.996 [§]			0.144 [§]
male	1 (ref)	-		1 (ref)	-	
female	0.85	0.26-2.73		1.56	0.86-2.82	
Donor gender			0.971 [§]			0.290 [§]
male	1 (ref)	-		1 (ref)	-	
female	0.98	0.35-2.75		0.72	0.40-1.32	
Gender mismatch			0.942 [§]			0.341 [§]
rec. M vs don. M	1 (ref)	-		1 (ref)	-	
rec. M vs don. F	0.83	0.42-1.67		0.64	0.29-1.42	
rec. F vs don. M	1.02	0.47-2.25		1.39	0.61-3.16	
rec. F vs don. F	1.0	0.42-2.42		1.08	0.43-2.74	
+49A>G recipient genotype			0.014 [*]			0.025 [*]
A/A	1 (ref)	-		1 (ref)	-	
A/G	0.67	0.40-1.10		0.53	0.29-0.97	
G/G	0.09	0.13-0.72		0.26	0.06-1.12	
+49A>G donor genotype			0.130 ^{§*}			0.610 ^{§*}
A/A	1 (ref)	-		1 (ref)	-	
A/G	1.28	0.75-2.19		1.12	0.52-2.40	
G/G	0.36	0.08-1.69		0.81	0.17-3.98	
Source of stem cells			0.324 [§]			0.492 [§]
peripheral blood	1 (ref)	-		1 (ref)	-	
bone marrow	0.76	0.45-1.35		1.28	0.63-2.61	
Disease type			0.369 [§]			0.309 [§]
acute lymphoid	1 (ref)	-		1 (ref)	-	
acute myeloid	0.48	0.19-1.17		0.39	0.14-1.05	
lymphoma	0.73	0.30-1.82		0.51	0.17-1.48	
myeloproliferative	0.48	0.19-1.23		0.44	0.15-1.28	
Disease stage			<0.001			0.011
early	1 (ref)	-		1 (ref)	-	
advanced	2.63	1.59-4.34		2.15	1.19-3.89	

TBI			0.160 [§]		<0.001
yes	1 (ref)	-		1 (ref)	-
no	1.46	0.86-2.45		2.84	1.55-5.24

In multivariate Cox regression analyses, all variables were initially included in the model. Variables not significantly associated with overall survival or disease-free survival ($p>0.05$) were removed from the model by means of a step-down procedure.

[¶] HR, hazard ratio

[#] 95% CI, 95% confidence interval

[‡] p value from likelihood ratio test

^{*} P value from a χ^2 test with 1 degree of freedom, assessing the increasing/decreasing trend of HR over the discrete levels of the covariate

[§] variable removed from the final model

Jurgen Finke, MD
Associate Editor
Annals of Hematology

December 2 , 2009

Dear Editor,

I am hereby submitting the third revised version of our manuscript Ref. No: AOHE-D-09-00069 entitled "CTLA-4 +49 A>G polymorphism of recipients of HLA-matched sibling allogeneic stem cell transplantation is associated with survival and relapse incidence".

As suggested, we have now addressed the minor comments of Reviewer #1 as follows. Modifications are underlined.

REVIEWER #1

Point 1

According to the Reviewer's request, we have indicated in the figure that the p values are from the log rank test. In the figure legend, we have also specified that the log rank test estimates differences in terms of OS or DFS among the 3 genotypes of Recipient considered as separate discrete categories (+49GG, AG, AA).

As requested we have included "*log rank p=0.027" and "*log rank p=0.036" at the bottom left of the OS and DFS graphics, respectively, in **Figure 1** and the following new sentence: "* the P values, obtained by log rank tests, refer to the differences in terms of OS and DFS across the 3 subgroupss of the discrete variable (recipient genotype)" in the **Legend to Figure 1**.

Point 2

In the last paragraph of **Results, page 8, line 1**, the word "response" has been deleted with the new sentence becoming: "In view of these results and of the known unfavorable effect of acute GvHD on both OS and DFS, we further investigated a possible correlation between acute, as well as chronic GvHD, and the +49A/G or G/G recipient genotype".

Point 3

Since this statistical analysis was not performed, we agree with the Reviewer that our sentence was too speculative. We thus have deleted the following sentence: "According to this hypothesis, the GvL effect in our series of patients was independent from the severity of GVHD" in the **Discussion Page 10, lines 8-9**.

We believe that we have addressed the minor concerns raised by the Reviewer #1 and hope that the revised manuscript will meet the requirements for publication in Annals of Hematology.

We thank you very much for your attention and look forward to hearing from you.

Yours sincerely,

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