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INFLUENCE OF CYTOTOXIC T LYMPHOCYTE-ASSOCIATED ANTIGEN 4 (*CTLA4*) COMMON POLYMORPHISMS ON OUTCOME IN TREATMENT OF MELANOMA PATIENTS WITH CTLA-4 BLOCKADE

Willemijn B Breunis[1,2,*], **Eduardo Tarazona-Santos**[1,3,*], **Renee Chen**[1,4], **Maureen Kiley**[1], **Steven A Rosenberg**[5], and **Stephen J Chanock**[1,4]

[1]Section on Genomic Variation, Pediatric Oncology Branch, National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD 20892-4605, USA [2]Emma Children's Hospital, Academic Medical Center, University of Amsterdam, The Netherlands [3]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627 – Pampulha, Caixa Postal 486, CEP 31270-910, Belo Horizonte, Minas Gerais, Brazil [4]Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4605, USA [5]Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Blockade of the Cytotoxic T Lymphocyte-associated Antigen 4 (CTLA-4), a down-regulator of T cell activation, can cause cancer regression in patients with metastatic melanoma. However, not all patients respond well to the therapy and some develop severe autoimmune reactions. We hypothesized that common genetic variation in the *CTLA4* gene could contribute to response to CTLA-4 blockade and the occurrence of autoimmune reactions. We investigated seven common single-nucleotide polymorphisms, SNPs, (rs733618, rs4553808, rs11571317, rs5742909, rs231775, rs3087243 and rs7565213) in 152 Caucasian melanoma patients who received CTLA-4 blockade. Three SNPs were associated with response to therapy: proximal promoter SNPs, rs4553808 (p=0.002, OR 3.39, 95%CI:1.62–7.10) and rs11571327 (p=0.02, OR 2.89, 95%CI: 1.23–6.83) and the non-synonymous SNP rs231775 (Thr17Ala, p=0.009, OR 0.39; 95% CI 0.18–0.82). A haplotype analysis including the 7 SNPs suggested that the common haplotype, TACCGGG could be associated with no response (p=0.02) whereas the haplotype TGCCAGG (p=0.06, OR: 4.13, 95%CI: 1.17–14.5) could be associated with response to the treatment. No significant association was observed for occurrence of severe autoimmune reactions (grade III/IV) either by single SNP or haplotype analyses. Our results suggest that genetic variation in *CTLA4* could influence response to CTLA-4 blockade therapy in metastatic melanoma patients, but further studies are necessary to confirm the observed associations.

Keywords

CTLA4; Single Nucleotide Polymorphism; CTLA-4 blockade; melanoma

Address all correspondence to: Stephen J. Chanock, Laboratory of Translational Genomics, National Cancer Institute, Advanced Technology Center, 8717 Grovemont Circle, Gaitherburg, MD 20892-4605, USA, Phone: (301)-435-7559, Fax: (301)-402-3134, sc83a@nih.gov.
*Both authors equally contributed to the present paper.

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INTRODUCTION

Clinical studies have shown that Cytotoxic T-lymphocyte-associated Antigen 4 (CTLA-4) blockade can cause cancer regression in patients with metastatic melanoma (1, 2). By blocking the action of CTLA-4, namely down-regulation of T cell activation, the immune response can be enhanced to increase anti-tumor activity. However, not all patients seem to benefit from the blockade treatment and response to the therapy is correlated with grade III/IV autoimmune reactions such as colitis and dermatitis (3). The mechanisms underlying the observed auto-immune reactions and inter-individual variability in response to therapy are not well understood (4).

Common genetic variants, known as single nucleotide polymorphisms (SNPs) in the *CTLA4* gene have been associated with a range of T cell mediated autoimmune diseases (5). In fact, selected SNPs in *CTLA4* have been reported to alter either expression or function of the gene product (6, 7). The new tools of genomics enable the testing of common genetic variants by an indirect method, in which well-chosen surrogates can monitor untested variants if they are in linkage disequilibrium (LD) (8). Thus, it is possible to utilize the pattern of LD within *CTLA4* to select a subset of SNPs (tag-SNPs) to investigate common genetic variation in this gene. Johnson et al. described 5 tag-SNPs for the *CTLA4* gene in an European population (9). Two other SNPs, known previously as CT60 (rs3087243) and JO30 (rs7565213) located downstream in the 3'UTR of the *CTLA4* gene have been associated with different auto-immune diseases (5). Here we report a preliminary study in patients with metastatic melanoma, treated with CTLA-4 blockade to investigate whether genetic variation at *CTLA4* is associated with response and with the occurrence of autoimmune reactions after therapy.

METHODS

One hundred and fifty-two patients of European ancestry with progressive stage IV melanoma were included. All subjects were treated at the Clinical Center of the National Institutes of Health on protocols approved by the Institutional Review Board and received treatment with serial i.v. administration of a fully human anti-CTLA-4 antibody (MDX-010). Characteristics of the majority of these patients and the treatment protocol have been described elsewhere (1).

Clinical response to the therapy was assessed following the Response Evaluation Criteria in Solid Tumors (RECIST, 10) guidelines, namely, we measured response within 4 weeks of starting treatment. Subsequently, after every two therapy cycles all patients underwent computed axial tomography of the chest, abdomen and pelvis and Magnetic Resonance Imaging of the brain. The sum of the largest diameters of all tumors in each patient was calculated before and after treatment. A decrease of more than 30% (but < 100%) of this sum was defined as Partial Response. Disappearance of all tumors for ≥ 1 month was considered a Complete Response. Autoimmune toxicity associated with therapy was classified as grade I/II or III/IV (1). The analysis grouped together all subjects because there was not an observed difference in response rates at varying doses of anti-CTLA4. Similarly, no difference was observed in patients who did or did not receive peptide vaccination. Accordingly, the analysis was not stratified on the basis of dosage or peptide vaccination.

DNA extraction and genotyping

Genomic DNA was isolated from buffy coats using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, US). Genotyping of the seven SNPs was performed by bidirectional re-sequencing of five regions of the *CTLA4* gene. Primer pairs were designed using the Primer3 software (11) and included the following primers (M13 tails are not denoted): forward 5'-CTTGCACCTTCTGCTCAT CC-3' and reverse 5'-

CTTTTCTGACCTGCCTGTTTTC-3' for rs733618 and rs4553808; forward 5'-TTTGGGTTGGCTTTTCTTTG-3' and reverse 5'-CAACCTCAAGCACTCAACT GAA-3' for rs11571317 and rs5742909; forward 5'-TTCGACGTAACAGCTAAACC-3' and reverse 5'-ACAAATGAAACCCAGGTAGGAG -3' for rs231775 in exon 1; forward 5'-ATCTGTGGTGGTCGTTTTCC-3' and reverse 5'-CTGAGAAAGCAGGC GGTAAG-3' for rs3087243; and forward 5'-CTTCCTGCTTCCACCTTGTC-3' and reverse 5'-AGGGGCAAAAAGGTGCTC-3' for rs7565213. Sequencing reactions were performed using Big Dye Version 3.0 (Applied Biosystems, Foster City, CA, US) and the following conditions were used: initial 96° C for 2 min followed by 25 cycles at 96°C for 10 s, 50°C for 10s and 60°C for 4 min. Sequence reactions were run on 3100 or 3730 Applied Biosystems sequencers (Applied Biosystems, Foster City, CA, US). We used the Sequencher software v. 4.0.5 (Gene Codes Corporation, Ann Arbor, MI, USA) to visually determine genotype callings with the requirements that the genotypes must be consistent in both directions (forward and reverse sequencing, 12).

Statistical analyses

Because only three patients showed Complete Response, for statistical analyses we considered the single category of Responders to include 20 Partial-Responders and 3 Complete-Responders. Furthermore, toxicity was coded on the basis of presence or absence of grade III/IV autoimmune reactions such as arthalgia, colitis, dermatitis, enterocolitis, enteritis, episcleritis, esophagitis, hepatitis, hypophysitis, hypopit, meningitis, nephritis and uveitis. For single SNPs analyses, we tested the association for allele frequencies of responders versus the non-reponders and similarly for the development of autoimmune reactions by the Fisher exact test, and calculated odds ratios and their 95% confidence intervals. For haplotype analyses, we tested the association with response and toxicity using the software Haplo.Stats (version 1.1.1 written for R 1.7.1). This method infers the pairs of haplotypes carried by each individual using an Expectation-Maximization algorithm and uses a Generalized Linear Model (GLM) for regression (13, 14), allowing the calculation of Odds Ratios for each haplotype as well as their confidence intervals. This method measures association using haplotype and has the following advantages: (i) it allows for different models of inheritance; (ii) it assesses significance taking into account uncertainty about haplotype inferences; (iii) it infers chromosome phase and haplotype frequencies, conditioning on all the observed data and estimated regression parameters and; (iv) it allows inclusion of non-genetics and environmental variables as covariates such as toxicity. Moreover, besides testing for association for each observed haplotype, the method also performs a global test of association (13).

RESULTS AND DISCUSSION

We studied 152 European-ancestry melanoma patients treated with a CTLA-4 antibody by genotyping five CTLA4 tag-SNPs plus two well described SNPs in the 3'UTR of the gene (5–7, 9). Of the 152 treated patients, 23 (15.1%) showed response to therapy. From the forty-eight patients that developed grade III/IV autoimmune toxicity, 18 were responders. The single SNPs analysis is presented in Table 1. Alleles G of rs4553808, T of rs11571317 and A of rs231775 are significantly associated with response. SNPs rs4553808 and rs11571317 are located in the promoter region of *CTLA4*, while rs231775 is a non synonymous Thr17Ala substitution in exon 1, which encodes for the cell membrane signal peptide. Allele frequencies were also compared between patients that developed autoimmune reactions of grade III/IV and those who did not (Table 2), but no significant difference was observed. On the other hand, independent of genetic variation in *CTLA4*, there is a strong positive association among response to the treatment and grade II–IV toxicity (Fisher exact test $P < 0.002$), as previously reported for these patients by Attia et al.

(3). Sanderson et al., hypothesized that the GG allele of rs7565213 (JO30), reported to have lower CTLA4 activity, correlates with a higher change of developing autoimmune symptoms and subsequently could be associated with improved prognosis (15). In their study of 19 patients, 3 out of 4 patients with the GG allele developed autoimmune symptoms. In our study, we did not confirm this hypothesis because we did not observe a significant association of the G allele of re7565213 (JO30) with the development of autoimmune reactions nor with a measurable response.

Eleven different haplotypes were inferred by the Expectation-Maximization algorithm of the *Haplo.Stats* software and six of them are common (Table 3). The four most common haplotypes have frequencies similar to other European populations (7, 9, 16). We performed two sets of haplotype analyses. First, based on the results observed by the SNPs analysis, we assessed if any combination of the alleles positively associated with response (i.e. rs4553808-G, rs11571317-T and rs231775-A) also show association with response. None of the studied individuals had the haplotype carrying the three alleles associated with response (GTA). Haplotypes A<u>TA</u> (Score statistic = 2.37, P = 0.02, OR: 2.69, 95%CI: 1.01–7.18) and in particular <u>G</u>C<u>A</u> (Score statistic = 3.54, P = 0.0001, OR: 4.04, 95%CI: 1.54–10.6), carry two alleles associated with response (i.e. underlined) and were also associated with response. Second, we performed haplotype analyses considering the entire set of 7 SNPs. The 7-SNPs haplotype distribution differed between Responders and Non-responders (Global score statistic = 19.41, df = 6, P = 0.0035). Table 3 shows the results for two association tests performed by *Haplo.Stats* for each of the inferred common haplotypes: a haplotype-score test and a GLM-approach assuming an additive model for the genetic effect. Only the score test indicates a negative association between haplotype 2 and response. In fact, the frequency of this haplotype is 34.5% among Non-Responders, but only 17.4% among Responders. Moreover, the association for haplotype 2 is consistent with results of the individual SNPs analyses: it bears the alleles A, C and G of rs4553808, rs11571317 and rs231775, and they are all associated with no-response. While the estimates for the odd ratios (OR) determined by the GLM approach suggest a possible, positive association between haplotype 4 and response to the therapy, the overall significance is borderline (P value by score test is 0.06 in this case). Since haplotype 4 has a frequency of 5.8% among Non-Responders and 13% among Responders, it is worthy of further study. These results did not change even after controlling for the effect of Grade III–IV toxicity (data not shown). Using the same analyses, no haplotype showed association with the presence of Grade III–IV toxicity (data not shown). On the other hand, we recognize the possibility that an association with stable disease could have been missed.

In our study, we employed an approach, based on haplotypes and tag-SNPs to optimally monitor common genetic variation across the *CTLA4* gene. We have used the tag-SNPs identified by Johnson et al. (9), which can monitor SNPs not directly genotyped in *CTLA4*. In this preliminary study, we provide preliminary evidence for association with three correlated SNPs and two *CTLA4* haplotypes with the response to treatment with anti-CTLA-4 antibodies in metastatic melanoma patients. Interestingly, one SNP is a non-synonymous SNP rs231775 (Thr17Ala) in exon 1, which encodes for the signal peptide of the protein. Anjos et al. (17) have shown by in vitro experiments that the *A(Thr)* allele is associated with a major expression on the cell surface than the *G(Ala)* allele. Although this is evidence that this polymorphism is functionally important and may be responsible for the observed association, an alternative explanation is that this SNP is in linkage disequilibrium with an untested *CTLA4* polymorphism, which actually could be functionally important. Our results are encouraging and suggest further investigation to confirm the association in a larger study as well as an investigation of the functional consequences of the associated variants. If the association is confirmed, it will be important to identify the common genetic variants or haplotypes in *CTLA4* responsible for the differential response to the treatment.

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Table 1

Allele frequencies (%) in responders and non responders.

SNP		Non responders (%)	Responders (%)	P	OR (95% CI)
−1721	rs733618				
T		237(92.6)	44(95.7)	0.54	0.54 (0.12–2.40)
C		19(7.4)	2(4.3)		
−1660	rs4553808				
A		225(88.7)	32(69.6)	0.002*	3.39 (1.62–7.10)*
G		29(11.4)	14(30.4)		
−657	rs11571317				
C		238(92.2)	37(80.4)	0.02*	2.89 (1.23–6.83)*
T		20(7.8)	9(19.6)		
−318	rs5742909				
C		245(95.0)	42(91.3)	0.32	1.71 (0.53–5.49)
T		13(5.0)	4(8.7)		
49	rs231775				
A (Thr)		150(58.6)	36(78.3)	0.009*	0.39 (0.18–0.82)*
G (Ala)		106(41.4)	10(21.7)		
CT60	rs3087243				
A		118(46.5)	22(47.8)	0.75	0.86 (0.46–1.61)
G		136(53.5)	24(52.2)		
JO30	rs7565213				
A		117(47.2)	26(56.5)	0.29	0.71 (0.39–1.29)
G		131(52.8)	20(43.5)		

OR = odds ratio, CI = confidence interval, P value obtained by Fisher exact test. We did not consider tests as independent because there is some level of linkage disequilibrium among SNPs.

*Significant results

Table 2

Allele frequencies (%) in patients with grade III/IV autoimmune reactions and patients with no autoimmune reactions.

SNP		Autoimmune reaction (%)	No autoimmune reaction (%)	P	OR (95% CI)
−1721	rs733618				
T		92(95.8)	189(91.7)	0.23	2.06 (0.67–6.32)
C		4(4.2)	17(8.3)		
−1660	rs4553808				
A		80(83.3)	177(86.8)	0.48	0.76 (0.39–1.49)
G		16(16.7)	27(13.2)		
−657	rs11571317				
C		83(86.5)	192(92.3)	0.14	0.53 (0.24–1.16)
T		13(13.5)	16(7.7)		
−318	rs5742909				
C		92(95.8)	195(93.8)	0.59	1.53 (0.48–4.83)
T		4(4.2)	13(6.2)		
49	rs231775				
A		64(66.7)	122(59.2)	0.25	1.37 (0.82–2.28)
G		32(33.3)	84(40.8)		
CT60	rs3087243				
A		47(49.0)	93(45.6)	0.62	1.14 (0.70–1.86)
G		49(51.0)	111(54.4)		
JO30	rs7565213				
A		50(52.1)	98(47.1)	0.46	1.22 (0.75–1.98)
G		46(47.9)	110(52.9)		

OR = odds ratio, CI = confidence interval, P value obtained by Fisher exact test.

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Table 3

Association among CTLA4 haplotypes and outcome in treatment of melanoma patients with anti-CTLA-4 blockade (SNPs: rs733618, rs4553808, rs11571317, rs5742909, rs231775, rs3087243, rs7565213)

Haplotypes	Non responders (%)	Responders (%)	Total	Haplotype score	P	GLM-Additive model OR LowCI-OR HighCI-OR		
1.TACCAAA	99(38.4)	16(34.8)	115(37.8)	−0.66	0.50	Reference to calculate ORs		
2.TACCGGG	89(34.5)	8(17.4)	97(31.9)	−2.30	0.02*	0.52	0.20	1.37
3.TATCAAA	20(7.8)	6(13.0)	26(8.6)	1.43	0.16	2.08	0.73	5.92
4.TGCCAGG	15(5.8)	6(13.0)	21(6.9)	1.85	0.06	4.13	1.17	14.50*
5.CACCGGG	18(7.0)	2(4.3)	20(6.6)	−0.68	0.55	0.75	0.14	3.85
6.TGCTAGG	13(5.0)	4(8.7)	17(5.6)	0.91	0.53	2.03	0.61	6.75
7. Rare	4(1.5)	4(8.6)	8(2.6)	-	-	-	-	-
	258	46	304					

*Signficant association. GLM: Generalized Linear Model. OR: Odds Ratio. CI: 95% confidence interval estimated using a GLM. “Rare” haplotypes include 5 different haplotypes with frequencies < 5%.