A polymorphism associated with *STAT3* expression and response of chronic myeloid leukemia to interferon α

Sebastian Kreil,[1,3] Katherine Waghorn,[1,2] Thomas Ernst,[1,3] Andrew Chase,[1,2] Helen White,[1] Rüdiger Hehlmann,[3] Andreas Reiter,[3] Andreas Hochhaus,[3] and Nicholas C.P. Cross[1,2] on behalf of the German CML Study Group

[1]Wessex Regional Genetics Laboratory, Salisbury District Hospital, Salisbury, UK; [2]Human Genetics Division, University of Southampton School of Medicine, Southampton, UK, and [3]III. Medizinische Universitätsklinik, Medizinische Fakultät Mannheim der Universität Heidelberg, Germany

ABSTRACT

Interferon α (IFN) induces variable responses in chronic myeloid leukemia (CML), with 8-30% of early chronic phase cases achieving a complete cytogenetic response. We hypothesized that polymorphic differences in genes encoding IFN signal transduction components might account for different patient responses. We studied 174 IFN-treated patients, of whom 79 achieved less than 35% Philadelphia-chromosome (Ph) positive metaphases (responders) and 95 failed to show any cytogenetic response (more than 95% Ph-positive metaphases; non-responders). We compared 17 single nucleotide polymorphisms (SNPs) at *IFNAR1, IFNAR2, JAK1, TYK2, STAT1, STAT3* and *STAT5a/b* between the two groups and found a significant difference for rs6503691, a SNP tightly linked to *STAT5a*, *STAT5b* and *STAT3* (minor allele frequency 0.16 for non-responders; 0.06 for responders, P=0.007). Levels of *STAT3* mRNA correlated with rs6503691 genotype (P<0.001) as assessed by real time quantitative PCR and therefore we conclude that rs6503691 is associated with the *STAT3* expression levels and response of CML patients to IFN.

Key words: *STAT3*, chronic myeloid leukemia, interferon.

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Introduction

Interferon α (IFN) induces heterogeneous responses in chronic myeloid leukemia (CML), with up to 80% of early chronic phase patients achieving hematologic remission but only 8-30% achieving complete cytogenetic remission.[1-6] Although response correlates with Hasford and Sokal risk scores[7,8] and may be influenced by other factors such as the presence or absence of deletions at the reciprocal *ABL/BCR* junction on the 9q+ chromosome,[9] the molecular basis for heterogeneous responses, and indeed more broadly the mechanism of response to IFN, remains poorly understood.

The type 1 IFN receptor is heterodimeric in structure, with the two subunits encoded by the genes *IFNAR1* and *IFNAR2*. Binding of IFN to the receptor induces activation of the JAK1 and TYK2 non-receptor tyrosine kinases which then phosphorylate STAT proteins.[10] Phosphorylated STAT dimers migrate to the nucleus where they activate the transcription of target genes. Inherited single nucleotide polymorphisms (SNPs) in genes encoding components of the IFN signal transduction cascade have been associated with diseases such as systemic lupus erythematosus, athsma and Crohn's disease.[11-13] We hypothesized that polymorphic difference in this cascade might account for the different responses of CML patients to IFN.

Design and Methods

Patient samples

Initially we studied 174 pre-treatment genomic DNA (gDNA) samples from BCR-ABL-positive CML patients receiving IFN as part of the German CML studies I-III.[3,14,15] Two patient groups were selected on the basis of availability of DNA and maximal response to therapy: *responders* (n=79) achieved a major (≤35% Philadelphia chromosome-positive metaphases) or complete cytogenetic response (0% Philadelphia chromosome-positive metaphases) whereas the *non-responders* (n=95) failed to show any cytogenetic response (>95% Philadelphia chromosome-positive metaphases) after a median of 38 and 22 months treatment, respectively, after initiation of treatment. Samples from an additional 245 pre-

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Correspondence: Nicholas C.P. Cross, Wessex Regional Genetics Laboratory, Salisbury District Hospital Salisbury, SP2 8BJ, UK. E-mail: ncpc@soton.ac.uk
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treatment CML cases for whom both DNA and cDNA were available were used to compare *STAT* expression levels with genotype. The study was approved by the Internal Review Boards from participating institutions and informed consent was provided according to the Declaration of Helsinki.

SNP genotyping by pyrosequencing

We studied 17 single nucleotide polymorphisms (SNPs) that were within or close to the genes encoding *IFNAR1, IFNAR2, JAK1, TYK2, STAT1, STAT3* and *STAT5a/b*. SNPs were selected on the basis of published data indicating positive associations with one or more human diseases, or as tagged SNPs with minor allele frequencies (maf) >0.2 from the International HapMap Project (release 21; *www.hapmap.org*). We did not include *STAT2* and *STAT4* in the analysis as there have not, to our knowledge, been any reports implicating these proteins in the pathogenesis of myeloid disorders. Furthermore, because of the limited number of cases available for analysis we deliberately did not attempt to capture all genetic variation at these loci due to the loss of statistical power this would entail. Pyrosequencing was performed as described[16] using primers and dispensation orders as shown in *Online Supplementary Tables S1A and B*. Markers were quantified using the Allele Frequency Quantification function in the SNP Software (Biotage AB, Uppsala, Sweden) and called as homozygous when one allele gave a reading of >90% and heterozygous when both alleles were called as 40-60%.

Expression analysis

Reverse transcriptase real-time PCR (RQ-PCR) was performed to quantify *STAT3, STAT5a* and *STAT5b* expression relative to GUSB expression and as an internal control for cDNA quality and quantity. Complementary DNA synthesis was performed by standard procedures and GUSB quantification was performed.[17] STAT3 expression was determined by using the custom designed PerfectProbe Gene Detection Kit (PrimerDesign, Southampton, UK) (sense primer: 5'-GAAGGAGGCGTCACTTTCAC-3'; antisense primer: 5'-CTGCT-GCTTTGTGTATGGTTC-3'; probe 5'FAM-CTCTTACCGCTGAT-GTCCTTCTCCACCCAGGTAAGAG-DABCYL3'). *STAT5a* and *STAT5b* expression was determined using the inventoried TaqMan® Gene Expression Assay by Applied Biosystems (Foster City, CA, USA). PCRs were performed on the Corbett Rotor-Gene 6000 (Corbett Life Science, Cambridge, UK). After demonstrating equal amplification efficiencies for each target, samples were tested in triplicate and mean *STAT* levels were normalized to *GUSB* and compared using the $2^{-\Delta Ct}$ method.[18]

Statistical analysis

To investigate the distribution of baseline values between groups, univariate tests were performed by using the Mann-Whitney, Fisher's exact or χ^2 tests, as appropriate. The possible independent influence of rs6503691 was assessed by multiple Cox regression analysis using SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA). Real time PCR results were compared to genotype by Kruskal-Wallis analysis.

Results and Discussion

Initially we genotyped 12 SNPs and compared the allele frequencies between responders and non-responders. As shown in Table 1A, only one SNP (rs6503691) in exon 1 of STAT5b showed a significant difference with a maf of

Table 1A. Summary of genotyping analysis.

Gene	IFNAR1	IFNAR1	IFNAR2	JAK1	JAK1	JAK1	STAT1	STAT3	STAT3	STAT5A	STAT5B	STAT5B	STAT5B	STAT5B	STAT5B	TYK2	TYK2
SNP	rs	rs	rs	rs	rs	rs	rs	rs	rs	rs	rs	rs	rs	rs	rs	rs	rs
	2850015	225716	77279064	29912	69310227	310229	1467199	6503695	2293152	2293154	16967611	6503691	9900213	1750023	17591972	2304256	12720356
Genotype	C/T	C/G	G/T	C/T	A/G	A/G	C/G	C/T	C/G	A/G	A/G	C/T	G/T	G/T	A/G	A/C	G/T
IFN responder; n=	73	72	74	73	72	64	79	79	79	79	79	79	79	77	77	74	74
Genotype A/A; n=	42	3	8	6	5	1	66	9	2	4	42	70	59	0	65	4	1
Genotype A/B; n=	26	22	33	19	17	8	7	34	28	14	29	8	18	10	11	28	10
Genotype B/B; n=	5	47	33	48	50	55	6	36	49	61	8	1	1 2	67	1	42	63
Allele A; n=	110	28	49	31	27	10	139	52	32	22	113	149	136	10	141	36	12
Allele B; n=	36	116	99	115	117	118	19	106	126	136	45	10	22	144	13	112	136
Frequency allele A[%]	75.3	19.4	33.1	21.2	18.8	7.8	88.0	32.9	20.3	13.9	71.5	93.7	86.1	6.5	91.6	24.3	8.1
Frequency allele B[%	24.7	80.6	66.9	78.8	81.3	92.2	12.0	67.1	79.7	86.1	28.5	6.3	13.9	93.5	8.4	75.7	91.9
IFN non-responder;	84	84	84	84	83	80	95	95	95	95	95	95	100	93	83	84	83
Genotype A/A; n=	42	1	6	5	1	2	68	12	0	7	42	70	73	0	62	9	1
Genotype A/B; n=	35	27	43	28	21	13	21	47	42	23	43	20	22	7	19	35	14
Genotype B/B; n=	7	56	35	51	61	65	6	36	53	65	10	5	5	86	2	40	68
Allele A; n=	119	29	55	38	23	17	157	71	42	37	127	160	168	7	143	53	16
Allele B; n=	49	139	113	130	143	143	33	119	148	153	63	30	32	179	23	115	150
Frequency allele A[%]	70.8	17.3	32.7	22.6	13.9	10.6	82.6	37.4	22.1	19.5	66.8	84.2	84.0	3.8	86.1	31.5	9.6
Frequency allele B [%]	29.2	82.7	67.3	77.4	86.1	89.4	17.4	62.6	77.9	80.5	33.2	15.8	16.0	96.2	13.9	68.5	90.4
P value	0.38	0.66	1.00	0.79	0.28	0.54	0.18	0.43	0.70	0.20	0.35	0.0066	0.66	0.32	0.16	0.17	0.69
Odds ratio	0.79	0.86	0.98	1.08	0.70	1.40	0.65	1.22	1.12	1.50	0.80	0.36	0.85	0.56	0.57	1.43	1.21
95% confidence interval	0.48-1.31	0.49-1.54	0.61-1.57	0.63-1.86	0.38-1.28	0.62-3.18	0.35-1.20	0.78-1.90	0.67-1.88	0.84-2.66	0.50-1.27	0.17-0.76	0.47-1.53	0.21-1.52	0.28-1.18	0.87-2.36	0.55-2.65

0.16 for non-responders versus 0.06 for responders (P=0.0066, odds ratio 0.36, 95% confidence intervals 0.17-0.76). Typing of an additional 5 SNPs in the same genomic region (rs6503695, rs16967611, rs9900213, rs17500235, rs17591972) failed to reveal any other significant associations (Table 1A). It is notable that this SNP has been recently reported to be associated with the risk of developing breast cancer.[19] We evaluated the impact of rs6503691 in more detail by taking other prognostic factors into account. On univariate analysis, the leukocyte count, percentage blasts, spleen size, Sokal score and rs6503691 genotype were all significantly associated with response (Table 1B). On multivariate analysis, however, rs6503691 genotype fell marginally below the level of significance (P=0.056; Table 1C).

Inspection of the HapMap data shows that rs6503691 falls in a region of strong linkage disequilibrium at 17q21 that includes the entire *STAT5A* gene as well as the 5' end of *STAT5B* and the 3' end of *STAT3* (Figure 1A). Potentially then, this SNP could be linked to other variants that might influence the expression of any of these three genes. We therefore compared rs6503691 genotype with *STAT5A, STAT5B* and *STAT3* mRNA levels in 245 pre-treatment CML cases. As shown in Figure 1B, *STAT3* expression was strongly related to rs6503691 genotype (P<0.0001) but no differences were seen for *STAT5A* or *STAT5B*. Strikingly, a nearby polymorphism has recently been reported to be linked to both *STAT3* mRNA levels and the response of metastatic renal cell carcinoma to IFN.[20] BCR-ABL is known to activate *STAT3*[21] and elevated expression of SOCS3, a known STAT3 target, confers IFN resistance to CML cells.[22] Taken together, these results indicate that polymorphic differences in *STAT3* expression levels may be a determinant of response to IFN in CML, and that the marginal lack of significance on multivariate analysis may have been due, at least in part, to limited sample numbers.

In the past decade, treatment of patients with CML has been transformed by the introduction of imatinib and other second generation tyrosine kinase inhibitors (TKIs). Nevertheless, IFN still remains relevant and its use as part of combination therapy with TKIs has attracted considerable interest, supported by favorable early clinical results

Table 1B. Clinical characteristics and univariate analysis of responders and non-responders.

Clinical characteristics [data given as median (range) or n (percent]	Total n=174	Non-responders n=95 (55%)	Responders n=79 (45%	*P* value*
Age in years	50 (10-83)	51 (17-83)	49 (10-76)	n. s.
Sex (% male)	103 (59)	54 (57)	49 (62)	n. s.
White blood cell count (×10⁹/L)	84 (6-475)	97 (19-475)	66 (6-471)	0.021
Blasts (% in peripheral blood)	1 (0-15)	1 (0-15)	0 (0-9)	< 0.001
Eosinophils (% in peripheral blood)	2 (0-10)	2 (0-10)	2 (0-9)	n. s.
Basophils (% in peripheral blood)	3 (0-40)	3 (0-20)	3 (0-40)	n. s.
Platelets (×10⁹/L)	371 (86-2343)	407 (126-2343)	356 (86-2020)	n. s.
Hemoglobin (g/dL)	12.3 (6.9-18.8)	12.0 (6.9-18.8)	12.7 (8.0-16.2)	0.012
Spleen enlargement (cm)	1 (0-30)	2 (0-30)	0 (0-17)	0.009
Sokal risk group (%)				0.033
low	76 (46)	34 (37)	42 (58)	
intermediate	54 (33)	34 (37)	20 (27)	
high	36 (21)	25 (26)	11 (15)	
Hasford risk group (%)				n. s.
low	72 (43)	37 (40)	35 (48)	
intermediate	79 (48)	44 (47)	35 (48)	
high	15 (9)	12 (13)	3 (4)	
Additional chromosome abnormality (% yes)	27 (18)	15 (19)	12 (16)	n. s.
Chromosome 9q+ deletion (% yes)	7 (4)	2 (2)	5 (6)	n. s.
rs6503691 (% genotype A/A)	140 (80)	70 (74)	70 (89)	0.0066

**P value when testing the hypothesis of equal distribution between patients with and without response (Fisher's or Mann-Whitney U test, as appropriate.*

Table 1C. Multiple logistic regression analysis.

Clinical characteristics in final model (n=174)	n (%)	Estimated coefficient	Standard deviation	*P* value	Estimated odds ratio	95% confidence interval lower limit	upper limit
Blasts (% > 0 in peripheral blood)	101 (58)	-1.246	0.340	< 0.001	0.288	0.148	0.560
Platelets (% > 600×10⁹/L)	41 (24)	-0.924	0.401	0.021	0.397	0.181	0.872
rs6503691 (% genotype A/A)	140 (80)	0.816	0.427	0.056	2.262	0.980	5.221

**Cases belonging to the indicated groups were coded by 1, otherwise by 0.*

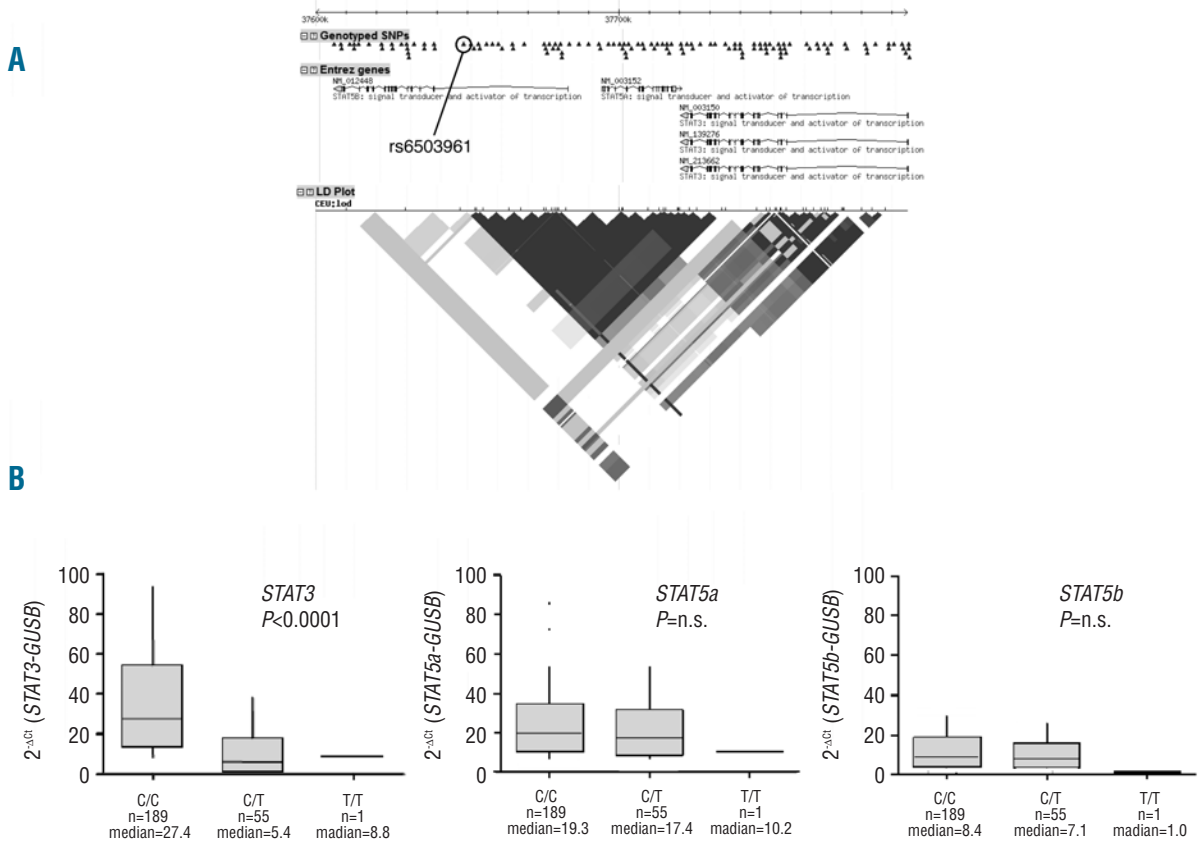

Figure 1. (A) HapMap data showing the position of rs6503961 in relation to *STAT5A*, *STAT5B* and *STAT3*, plus linkage disequilibrium in this region. (B) *STAT3* but not *STAT5A* or *STAT5B* expression normalized to *GUSB* is significantly related to rs6503691 genotype in 245 patients with CML (*P*=<0.0001; Kruskall-Wallis test). n.s = not significant.

and the recent demonstration that IFN activates dormant hemopoietic stem cells *in vivo*.[23] Furthermore, discontinuation of imatinib in cases that have achieved complete molecular remission (CMR) does not always lead to relapse, and it has been suggested that sustained CMR may be influenced by prior treatment with IFN.[24] We suggest that the impact of rs6503691 should be evaluated in these novel settings.

Authorship and Disclosures

SK, AH and NC designed the study. SK, KW, TE, AC, HW designed and performed the laboratory analysis. RH, AR and AH provided samples and clinical data. SK and NC analyzed the data. SK and NC wrote the paper, and all authors contributed to the final version.

The authors reported no potential conflicts of interest.

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