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Predictive Biomarkers and Personalized Medicine

Clinical Cancer Research

The *IFNG* (IFN-γ) Genotype Predicts Cytogenetic and Molecular Response to Imatinib Therapy in Chronic Myeloid Leukemia

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Abstract

Purpose: The present study analyzed treatment outcomes of imatinib therapy by interindividual genetic variants in candidate biological pathways of chronic myeloid leukemia (CML) such as apoptosis, angiogenesis, IFN-γ signaling pathways, or drug transport/metabolism of imatinib.

Experimental Design: Peripheral blood DNAs were genotyped for 79 single nucleotide polymorphism markers involved in the pathways of apoptosis, angiogenesis, myeloid cell growth, xenobiotic metabolism, *WT1* signaling, IFN signaling, and others in CML patients who were included in discovery (n = 229, Canada) and validation cohorts (n = 187, Korea).

Results: We found several genotypes associated with complete cytogenetic response: *IFNG* (rs1861494, rs2069705), *FASL* (rs763110), *FAS* (rs2234767, rs2234978), *VEGFR2* (rs1531289), and *WT1* (rs2234590); with major molecular response: *IFNG* (rs1861494, rs2069705), *BIRC5* (rs9904341), *FAS* (rs2234978), and *ABCG2* (rs2231142); with loss of response: *IFNG* (rs2069705), *IFNGR2* (rs9808753), *BIRC5* (rs9904341), and *ORM* (rs3182041); and with treatment failure: *IFNG* (rs2069705), *JAK3* (rs3212713), and *ORM* (rs3182041). External validation for the above significant genotypes confirmed that the *IFNG* genotype (rs2069705) was predictive of complete cytogenetic response (hazard ratio, 2.17; $P < 0.001$) and major molecular response (hazard ratio, 1.96; $P = 0.0001$) in validation cohorts of Korean ethnicity.

Conclusions: The *IFNG* genotype was predictive for response to imatinib therapy, suggesting potential involvement of the IFN-γ signaling pathway in the mechanism of action of imatinib in CML. *Clin Cancer Res; 16(21); 5339–50. ©2010 AACR.*

Imatinib is a selective tyrosine kinase inhibitor, particularly against BCR/ABL fusion tyrosine kinase, and provides therapeutic benefit to patients with chronic myeloid leukemia (CML). However, interindividual variability of response to imatinib exists (1). For investigation of interindividual variation in drug response or resistance to imatinib, an approach using single nucleotide polymorphisms (SNP) is particularly worthwhile. In the present study, we simultaneously examined multiple candidate gene SNPs in terms of their association with imatinib response and development of resistance in patients with CML.

The Abelson tyrosine kinase domain mutation is an important mechanism in imatinib resistance. However, a substantial proportion of patients with imatinib resistance do not show evidence of tyrosine kinase domain mutation. Accordingly, other mechanisms aside from Abelson tyrosine kinase domain mutation should be explored intensively. Once imatinib binds to the bcr/abl oncoprotein, it then inactivates signal transduction, leading to cell death or apoptosis. Findings from recent studies have revealed that alternative pathways, such as the src kinase–mediated pathway, are switched on when imatinib blocks the bcr/abl-mediated pathway; thus, CML cells escape cell death

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The significance of leukemic stem cells has been increasingly emphasized by recent investigations. The current study attempted to investigate multiple candidate gene single nucleotide polymorphisms associated with treatment outcome of imatinib for chronic myeloid leukemia (CML). One of the interesting results was that the response to imatinib was associated with *IFNG* gene single nucleotide polymorphisms involved in the IFN signaling pathway which, until recently, had been known to be associated with hematopoietic stem cell proliferation and quiescence. Depending on the individual's genetic variability in expressing IFN-γ, the expansion and proliferation of hematopoietic stem cells could vary, thus affecting exposure to imatinib therapy. Based on the results from the current study, which was confirmed by internal validation using Bootstrap method and by external validation in an independent cohort of CML patients, the *IFNG* genotype was found to be a predictive surrogate for response to imatinib therapy, suggesting the potential involvement of IFN signaling pathways in leukemic stem cell proliferation in the mechanism of action of imatinib in CML.

(2). Therefore, SNPs in the apoptosis pathway need to be evaluated in terms of the response and resistance to imatinib therapy in CML.

Besides the apoptosis pathway, other potential candidate pathways include angiogenesis pathways, cell growth pathways, *WT1* (Wilms tumor gene) pathways, drug transport/metabolism pathways, or IFN signaling pathways. In the current study, we included several SNPs in these pathways with the following evidence: (*a*) resistance to apoptosis is regulated by the expression levels of bcr/abl fusion tyrosine kinase (3, 4); (*b*) increasing angiogenesis and higher vascular endothelial growth factor (VEGF) production was noted in CML patients (5–7); (*c*) CML that are characterized by clonal proliferation and growth factor–independent myeloid cell growth such as *CSF2* (*G-CSF*; ref. 8), *CSF3* (*GM-CSF*; refs. 9, 10), *FLT3* (11), *JAK3* (12, 13), and *IL1* (14); (*d*) the WT1 gene may be involved in leukemogenesis of CML (15); (*e*) *ABCB1* (multidrug resistance-1; refs. 16–18) and *ABCG2* (breast cancer resistance protein) is highly expressed in $CD34^+$ progenitor cells in CML patients (19), and α1 acid glycoprotein levels could reflect pharmacologic resistance to imatinib in patients with CML during blastic phase (20); and (*f*) IFN affects the proliferation of hematopoietic stem cells (HSC) and affects myeloid colony formation in both murine and human models (21–24).

The SNP of certain genes might affect the promoter expression of the gene, thus controlling mRNA transcription speed, or result in amino acid residue change, thus switching the functions of the corresponding protein. These SNPs could result in interindividual variability in the gene expression of certain genes. Accordingly, investigations of SNPs on the multiple candidate pathways might be helpful to discover certain SNP markers predicting treatment outcomes of imatinib therapy in CML. In the present study, 79 SNPs in multiple candidate pathways, including 6 major pathways (i.e., apoptosis, angiogenesis, myeloid cell growth, multidrug resistance, *WT1* signaling pathways, and IFN signaling pathways) were evaluated in a discovery cohort (n = 229), and significant SNPs were validated in an independent validation cohort (n = 187).

Materials and Methods

Study population

The discovery cohort included 229 CML patients who began imatinib therapy between August 2000 and December 2006 at the Princess Margaret Hospital, Toronto, ON, Canada. The validation cohort included 187 CML patients who started imatinib therapy between March 2002 and December 2008 at the Samsung Medical Center, Seoul, Korea or at the Chonnam National University Hwasun Hospital, Hwasun, Korea. Patients started imatinib at doses of 400, 600, or 800 mg, depending on their disease status. Before or during imatinib therapy, blood samples were collected after informed consent was obtained from patients in accordance with the Declaration of Helsinki. The current study was approved by the Research Ethics Board of the University Health Network, University of Toronto, ON, Canada.

Sequenom MassARRAY genotyping system

Candidate genotypes were selected through review of the literature and by selection of SNPs in nonsynonymous SNPs in exon regions with a minor allele frequency of >0.05 (Table 1). If the frequency was not available, it was reported from the Entrez SNP site (http://www.ncbi.nlm.nih.gov/sites/entrez).

First, genotyping was undertaken with discovery cohort samples using the Sequenom iPLEX platform (http://www.sequenom.com/; Sequenom, Inc.). DNA was extracted using the Puregene DNA Purification Kit (Gentra Systems, Inc.). Detection of SNPs was performed by analysis of primer extension products generated from previously amplified genomic DNA using a Sequenom chip-based matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry platform. The details of the primers are described in Supplementary Table S2. Ninety six-well plates containing 2.5 ng of DNA in each well were amplified by PCR following the specifications of Sequenom. Unincorporated nucleotides in the PCR product were deactivated using shrimp alkaline phosphatase. Allele discrimination reactions were conducted by the addition of extension primer(s), DNA polymerase, and a cocktail mixture of deoxynucleotide triphosphates and dideoxynucleotide triphosphates to each well. MassExtend clean resin (Sequenom) was added to the mixture for removal of extraneous salts that might interfere with MALDI-TOF analysis. Primer extension products were then cleaned

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and spotted onto a SpectroChip. Genotypes were determined by spotting an aliquot of each sample onto a 384 SpectroChip (Sequenom), which was subsequently read by MALDI-TOF mass spectrometer. Duplicate samples and negative controls were included for evaluation of genotyping quality.

Following primary statistical analyses, significant genotypes identified as having an association with clinical outcome in the discovery cohort were processed for internal and external validation. For external validation, 26 of the significant and neighboring genotypes were genotyped in the validation cohort (n = 187) using the Sequenom iPLEX platform. Genotyping was processed at the Analytical Genetics Technology Centre, University Health Network, Toronto, Ontario, Canada (for discovery), and at Bioneer Inc., Chungwon, Korea (for external validation).

Evaluation and disease monitoring

Prior to the commencement of imatinib therapy, complete blood count and standard baseline biochemistry tests were performed with bone marrow evaluation for morphology, conventional cytogenetic analysis, and *BCR/ABL* mRNA reverse transcription-PCR. Cytogenetic analysis was performed by the G-banding technique. Patients were monitored regularly on an outpatient basis as follows: biweekly blood counts and biochemistry were obtained during the first month of imatinib therapy, and then monthly until a cytogenetic response was achieved, and then every 3 months thereafter. Until complete cytogenetic response (CCR) was confirmed, bone marrow evaluation and/or fluorescence *in situ* hybridization studies were performed every 3 months. Using quantitative *BCR/ABL* mRNA PCR, quantification of peripheral blood *BCR/ABL* fusion gene transcripts was repeated every 3 to 4 months regardless of cytogenetic response.

Peripheral blood samples (5 mL) were also analyzed using quantitative PCR for determination of levels of *BCR/ABL* fusion gene transcripts, according to the instructions of the manufacturer (ABI 9700 Thermal Cycler; Applied Biosystems) and following recommendations established for the standardization of this procedure at the international level (25–27). *BCR/ABL* transcript levels were measured and presented using the international scale. Nested PCR techniques were used for confirmation of results in selected samples with undetectable *BCR/ABL* transcript levels. Abl tyrosine kinase domain mutations were screened in any patient in an advanced phase of disease. For patients with the chronic phase (CP) of the disease beginning treatment with imatinib, mutation screening was indicated if response was not acceptable, or if any sign of loss of response (LOR) or progression was observed.

Definition of response criteria and end points

Response criteria were the same as previously defined in studies using imatinib (1, 28, 29). Briefly, cytogenetic responses were categorized as complete (CCR; 0% Ph^+ cells in marrow by conventional cytogenetics or fluorescence *in situ* hybridization), partial (1-34% Ph^+ cells in marrow), or minor (35-65% Ph^+ cells in marrow). A major cytogenetic response (MCR) was defined as the sum of CCR and partial cytogenetic response. Major molecular response (MMR) was defined as <0.1% of the *BCR/ABL* fusion gene transcript level on an international scale by quantitative PCR.

Time to treatment failure (TF) was defined as the interval between initiation of imatinib therapy and the occurrence of events, i.e., imatinib failure, including primary and secondary resistance, such as LOR. Time to LOR was defined as the interval between the date of any confirmed response and the date at which criteria for responses were no longer being met including (*a*) transformation from CP to accelerated phase (AP) or blastic crisis (BC); (*b*) loss of CCR/MCR, and (*c*) development of the Abelson tyrosine kinase domain mutation.

Time to transformation-free survival was defined as the interval between initiation of imatinib therapy and confirmation of progression to AP or BC, or death from any cause, whereas overall survival was calculated from the initiation of imatinib therapy until the date of death from any cause or the date of the last follow-up.

Statistical analysis

The SNPs genotyped in the discovery cohort were primarily evaluated for adequacy of Hardy-Weinberg equilibrium using χ^2 test. Genotype errors and genotype frequencies were summarized using Haploview version 3.32 (Broad Institute, Cambridge, MA; available at http://www.broad.mit.edu/mpg/haploview). The SNP *ABCB1* (rs2032582) had more than two alleles and was coded in two ways: (*a*) GG, G/–, and –/–; and (*b*) T/T, T/–, and –/–.

Cumulative incidences of MCR, CCR, and MMR were calculated with consideration for discontinuance of imatinib as competing risks for interest events. Probability of freedom from LOR and from TF was estimated and plotted using the Kaplan-Meier method. Probabilities of overall and transformation-free survival were also estimated using the Kaplan-Meier method. In univariate analyses, according to the 79 genotypes in genotype models, MCR, CCR, and MMR were compared using Gray test, and LOR, TF, transformation-free survival, and overall survival were compared using log rank test.

For validation of the genetic effect, we performed internal validation procedures using a bootstrap algorithm, and constructed the bootstrap confidence interval (CI). We applied bootstrap based on 500 replications. Results were obtained using the PROC SURVEYSELECT procedure in SAS version 9.1, and presented as the bootstrap hazard ratio (HR) CIs of the genetic effects adjusted by clinical factors. External validation was also performed using an independent validation cohort. Treatment outcomes were also compared according to significant genotypes using the log rank test, and adjusted by Bonferroni correction. Procedures were repeated in additive, dominant, and recessive models with and without Bonferroni's correction.

Next, multivariate analysis was performed using variables that were significantly associated with CCR and MMR, including the disease stage (CP versus AP/BC), the presence of additional cytogenetic abnormality, and *IFNG*

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Table 1. Summary of 79 candidate gene SNPs with univariate analyses of treatment outcomes

Gene	SNP ID	Gene description	Chromosome	MAF	Allele (m/M)	Call rate (%)	Univariate analyses (*P*)			
							CCR	MMR	LOR	TF
Apoptosis pathway (*n* = 32)										
BCL2	rs1801018	B-cell CLL/lymphoma 2	18	0.36	G/A	99.6	0.574	0.385	0.342	0.433
BCL2	rs2279115	B-cell CLL/lymphoma 2	18	0.46	A/C	98.7	0.706	0.706	0.055	0.102
BAX	rs11667351	BCL2-associated X protein	19	0.13	G/T	98.3	0.257	0.644	0.846	0.971
BCL2L2	rs7042474	BCL2-like 2	9	0.14	T/C	98.3	0.615	0.842	0.345	0.144
BCL6	rs1056932	B-cell CLL/lymphoma 6	3	0.33	C/T	98.3	0.222	0.803	0.688	0.870
BCL6	rs11545363	B-cell CLL/lymphoma 6	3	1.00	C	98.7	—	—	—	—
BCL2L11	rs6746608	BCL2-like 11	2	0.47	A/G	99.1	0.691	0.301	0.455	0.678
BCL2L11	rs12613243	BCL2-like 11	2	0.08	C/T	98.3	0.379	0.574	0.462	0.252
BIRC4	rs28382722	X-linked inhibitor of apoptosis	X	1.00	C	99.6	—	—	—	—
BIRC4	rs5956583	X-linked inhibitor of apoptosis	X	0.37	C/A (Q→P)	99.6	0.500	0.489	0.655	0.505
BIRC5	rs9904341	Survivin	17	0.35	C/G	98.3	0.122	0.003	0.050	0.515
BIRC5	rs2071214	Survivin	17	0.05	G/A (E→K)	99.1	0.656	0.799	0.232	0.383
CASP1	rs580253	Caspase 1	11	0.17	T/C	98.7	0.883	0.973	0.606	0.676
CASP3	rs1049253	Caspase 3	4	0.17	T/C	98.7	0.094	0.153	0.525	0.252
CASP7	rs7922608	Caspase 7	10	0.28	G/T	99.1	0.400	0.262	0.735	0.873
CASP8	rs1045485	Caspase 8	2	0.11	C/G (D→H)	99.6	0.989	0.175	0.989	0.781
CASP8	rs3769818	Caspase 8	2	0.28	T/C	98.3	0.725	0.677	0.208	0.200
CASP8	rs3834129	Caspase 8	2	0.43	Del/CTTACT	100	0.465	0.908	0.190	0.243
CASP9	rs4645981	Caspase 9	1	0.28	T/C	98.3	0.371	0.974	0.916	0.682
CASP10	rs13006529	Caspase 10	2	0.42	A/T (L→I)	99.6	0.182	0.591	0.206	0.352
CASP10	rs13010627	Caspase 10	2	0.06	A/G (V→I)	98.3	0.645	0.853	0.816	0.651
FASLG	rs763110	Fas ligand (TNF superfamily 6)	1	0.38	T/C	98.3	0.011	0.226	0.612	0.238
FAS	rs2234767	Fas (TNF receptor superfamily 6)	10	0.14	A/G	98.3	0.033	0.285	0.340	0.141
FAS	rs1800682	Fas (TNF receptor superfamily 6)	10	0.50	C/T	98.7	0.128	0.391	0.356	0.154
FAS	rs2234978	Fas (TNF receptor superfamily 6)	10	0.31	T/C	98.3	0.054	0.003	0.149	0.208
FAS	rs3218612	Fas (TNF receptor superfamily 6)	10	0.03	G/A	98.3	0.493	0.721	0.733	0.574
FAS	rs3218619	Fas (TNF receptor superfamily 6)	10	0.01	A/G (A→T)	98.3	0.920	0.338	0.358	0.296
APAF1	rs1439123	Apoptotic peptidase activating factor 1	12	0.19	C/T	98.3	0.344	0.125	0.493	0.244
APAF1	rs2288713	Apoptotic peptidase activating factor 1	12	0.12	G/T	99.6	0.171	0.055	0.210	0.141
TNFR2	rs1061622	Tumor necrosis factor receptor superfamily, member 1B	1	0.21	G/T (M→R)	98.7	0.936	0.663	0.534	0.852
PDCD1	rs2227981	Programmed cell death 1	2	0.38	T/C	98.7	0.631	0.513	0.503	0.927
GZMB	rs7144366	Granzyme B	14	0.40	T/C	99.6	0.275	0.186	0.706	0.910
Angiogenesis (*n* = 7)										
VEGFA	rs699947	Vascular endothelial growth factor A	6	0.46	A/C	99.1	0.846	0.784	0.498	0.580

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Table 1. Summary of 79 candidate gene SNPs with univariate analyses of treatment outcomes (Cont'd)

Gene	SNP ID	Gene description	Chromosome	MAF	Allele (m/M)	Call rate (%)	Univariate analyses (*P*)			
							CCR	MMR	LOR	TF
VEGFA	rs833061	Vascular endothelial growth factor A	6	0.46	C/T	99.1	0.817	0.866	0.295	0.416
VEGFA	rs2010963	Vascular endothelial growth factor A	6	0.30	C/G	93.4	0.809	0.718	0.060	0.291
VEGFA	rs3025039	Vascular endothelial growth factor A	6	0.13	T/C	98.3	0.103	0.773	0.604	0.604
VEGFR2	rs1531289	VEGF receptor 2	4	0.28	A/G	98.3	0.018	0.455	0.503	0.157
VEGFR2	rs1870377	VEGF receptor 2	4	0.26	A/T (Q→H)	98.3	0.136	0.240	0.295	0.091
VEGFR2	rs2305948	VEGF receptor 2	4	0.10	T/C (V→I)	98.3	0.557	0.696	0.950	0.723
Myeloid growth (*n* = 13)										
FLT3	rs35602083	fms-related tyrosine kinase 3	13	0.02	T/C (D→N)	98.3	0.079	0.521	0.332	0.186
CSF3	rs25645	G-CSF	17	0.40	A/G (L→L)	99.1	0.658	0.112	0.750	0.381
CSF3	rs1042658	G-CSF	17	0.29	T/C	99.6	0.186	0.437	0.738	0.619
CSF2	rs25882	GM-CSF	5	0.28	C/T (I→T)	99.6	0.949	0.903	0.841	0.559
JAK3	rs3008	Janus kinase 3	19	0.43	C/T	97.8	0.937	0.262	0.734	0.856
JAK3	rs3212713	Janus kinase 3	19	0.35	A/G	99.1	0.193	0.066	0.156	0.032
IL1A	rs17561	Interleukin 1α	2	0.28	T/G (A→S)	99.6	0.810	0.098	0.517	0.899
IL1A	rs1800587	Interleukin 1α	2	0.30	T/C	99.6	0.966	0.104	0.360	0.680
IL1B	rs1143634	Interleukin 1β	2	0.20	T/C	100	0.903	0.896	0.821	0.609
IL1B	rs1143633	Interleukin 1β	2	0.36	A/G	98.3	0.519	0.195	0.232	0.378
IL1B	rs1143627	Interleukin 1β	2	0.41	C/T	99.6	0.836	0.815	0.706	0.930
IL1B	rs16944	Interleukin 1β	2	0.40	A/G	99.6	0.837	0.819	0.554	0.910
IL1R	rs2228139	Interleukin 1 receptor, type I	2	0.30	T/C (A→G)	99.1	0.887	0.242	0.186	0.559
WT1 signaling (*n* = 7)										
WT1	rs1799937	Wilms tumor 1	11	0.29	C/T	98.3	0.485	0.768	0.676	0.477
WT1	rs2234590	Wilms tumor 1	11	0.01	G/A	99.1	0.033	0.055	0.356	0.264
WT1	rs2234591	Wilms tumor 1	11	0.002	G/A	98.3	0.917	0.440	0.506	0.458
WT1	rs2301250	Wilms tumor 1	11	0.44	T/C	99.6	0.663	0.455	0.879	0.821
WT1	rs2301252	Wilms tumor 1	11	0.44	T/G	98.3	0.667	0.399	0.880	0.880
WT1	rs2301254	Wilms tumor 1	11	0.44	C/T	99.6	0.663	0.455	0.879	0.821
WT1	rs6508	Wilms tumor 1	11	0.07	A/G	99.6				
Xenobiotic metabolism (*n* = 12)										
ABCB1	rs1045642	Multidrug resistance 1	7	0.50	C/T	98.3	0.450	0.847	0.770	0.847
ABCB1	rs2032582	Multidrug resistance 1	7	0.47	A, T/G (S→T/A)	99.6	0.950	0.883	0.481	0.845
ABCB1	rs1128503	Multidrug resistance 1	7	0.47	T/C	99.6	0.901	0.826	0.397	0.863
ABCG2	rs2231142	Breast cancer resistance protein	4	0.11	A/C (G→K)	99.6	0.347	0.008	0.632	0.267
CYP3A5	rs28383469	Cytochrome P450, family 3, A5	7	0.004	T/C (G→fs)	99.1	—	—	—	—
CYP3A5	rs28383468	Cytochrome P450, family 3, A5	7	1.00	G (H→Y)	98.3	0.426	0.737	0.688	0.980
OCT1	rs1867351	Solute carrier family 22, member 1	6	0.25	G/A	98.7	0.360	0.767	0.358	0.398
OCT1	rs12208357	Solute carrier family 22, member 1	6	0.05	T/C (R→C)	99.6	0.882	0.786	0.183	0.361

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							CCR	MMR	LOR	TF
OCT1	rs2282143	Solute carrier family 22, member 1	6	0.02	T/C (P→L)	98.3	0.999	0.597	0.737	0.791
OCT1	rs628031	Solute carrier family 22, member 1	6	0.37	A/G (M→V)	97.8	0.273	0.448	0.607	0.653
IFN signaling (*n* = 4)										
IFNG	rs1861494	IFNγ	12	0.26	C/T	99.6	0.003	0.001	0.194	0.154
IFNG	rs2069705	IFNγ	12	0.36	C/T	96.9	0.009	0.006	0.031	0.015
IFNGR1	rs3799488	IFNγ receptor 1	6	0.11	C/T	99.6	0.411	0.337	0.394	0.298
IFNGR2	rs9808753	IFNγ receptor 2	21	0.21	G/A (Q→R)	98.7	0.310	0.542	0.042	0.019
Others (*n* = 6)										
ORM	rs1126724	Orosomucoid 1	9	1.00	G (V→L)	97.8	—	—	—	—
ORM	rs3182034	Orosomucoid 1	9	1.00	C (R→C)	99.6	—	—	—	—
ORM	rs3182041	Orosomucoid 1	9	0.004	G/A (K→R)	99.1	0.234	0.488	0.000	0.000
GNB3	rs5443	G proteinβ polypeptide 3	12	0.36	T/C	98.3	0.152	0.845	0.912	0.195
ULK3	rs2290573	Unc-51–like kinase 3	15	0.34	T/C	95.2	0.755	0.887	0.955	0.771
PTK2	rs4554515	PTK2 protein tyrosine kinase 2	8	0.49	T/G	98.3	0.446	0.379	0.560	0.842

Abbreviations: NA; not available; MAF, minor allele frequency.

genotypes (rs2068705; CC versus CT/TT genotype). Multivariate analyses using Cox proportional hazard models were conducted using backward stepwise modeling and a *P* value for the likelihood ratio test of >0.05. HRs and 95% CIs were also estimated.

All statistical tests were two-sided, with the significance level set as 0.05, unless otherwise stated. Statistical data were obtained using an SPSS software package (SPSS 13.0, Inc.) and SAS version 9.1 (SAS Institute). Incidence curves were obtained using R package, version 2.4.1 (available at http://cran.r-project.org/).

Results

Demographic and disease characteristics and treatment outcomes

Out of 229 patients, 203 patients (89%) were in CP, 23 patients (10%) were in AP, and 3 patients (1%) were in BC in the discovery cohort, whereas 163 patients (90%) were in CP, 11 patients (6%) were in AP, and 7 patients (4%) were in BC in the validation cohort. Demographic and disease characteristics of the discovery and validation cohorts are described in Table 2, including age, gender, race, previous treatment prior to imatinib, cytogenetics, and dosage of imatinib. Except ethnicity and treatment prior to imatinib, two cohorts showed similar characteristics of disease.

In the discovery cohort, with a median duration of imatinib administration of 40.8 months (range, 1-86), the cumulative incidence of HR was 96% (95% CI, 92-99%) at 3 months. The cumulative incidence of MCR and CCR was 86% (81-91%) and 62% (56-69%) at 12 months after initiation of imatinib therapy, respectively. In the case of MMR, it was 33% (27-40%) and 52% (45-59%) at 1 and 2 years, respectively. Forty-six cases (27%) of TF were documented due to either resistance ($n = 38$) or intolerance ($n = 8$). Probability of freedom from LOR was 71% (63-78%) at 2 years, and 60% (51-69%) at 3 years after achievement of any response to imatinib therapy. Probability of freedom from TF was 69% (62-76%) and 58% (51-65%) at 2 and 3 years after initiation of imatinib therapy, respectively. The 5-year probability of transformation-free survival and overall survival was 90% (87-94%) and 95% (92-99%), respectively. As shown in Supplementary Table S2, treatment outcomes in the validation cohort were similar to those in the discovery cohort.

Results of univariate analyses in the discovery cohort

Detailed information and frequency of candidate SNPs are summarized in Table 1. Among them, five were excluded from the analysis because they were monomorphic in the current population: *CASP9* (rs4645981), *CYP3A5* (rs28383469), *ORM* (rs1126724), *ORM* (rs3182034), and *BCL6* (rs11545363).

All results of univariate analyses are presented in Table 1. With respect to the probability of achieving CCR, the following SNPs showed significant correlation; *IFNG* (rs1861494 and rs2069705; Fig. 1A), *FASL* (rs763110), *FAS* (rs2234767), and *VEGFR2* (rs1531289). When we divided *IFNG* (rs2069705) into CC versus CT/TT, the median time to CCR was 183 ± 36 days in the CC genotype group versus 273 ± 37 days in the CT/TT genotype ($P = 0.003$).

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The probability of achieving MMR was significantly associated with *IFNG* (rs1861494 and rs2069705; Fig. 1B), *BIRC5* (rs9904341), and *FAS* (rs2234978). When comparing MMR between groups with the CC genotype and the CT/TT genotype of *IFNG* (rs2069705), significant differences were also observed in favor of the CC genotype (median time to MMR; 358 ± 76 d in the CC genotype versus 774 ± 83 d in the CT/TT genotype; $P = 0.002$).

In terms of LOR, *IFNG* (rs2069705), *IFNGR2* (rs9808753), *BIRC5* (rs9904341) and *ORM* (rs3182041) showed significant correlation, whereas TF was strongly associated with *IFNG* (rs2069705), *JAK3* (rs3212713), and *ORM* (rs3182041).

Internal validation using the bootstrap method

For confirmation of these results, we performed an internal validation. Results were presented with the bootstrap HR CIs of the genetic effects, as shown in Table 3. *IFNG* (rs1861494 and rs2069705), *CASP9* (rs4645981), *CASP8* (rs3834129), *FASL* (rs763110), and *FAS* (rs2234767) were useful for the prediction of achievement of CCR, and *IFNG* (rs2069705), *FAS* (rs2234767), and *JAK3* (rs3212713) were helpful for MMR, whereas *IFNG* (rs2069705) and *BRIC5* (rs9904341) were useful for LOR and TF. Accordingly, our results from univariate analyses were internally validated.

External validation in an independent validation cohort

External validation was repeated for confirmation of these results using an independent external cohort derived from CML patients in Korea. Interestingly, among various SNPs with significance in univariate analyses

Table 2. Patients and disease characteristics in discovery and validation cohorts

	No. of patients (%)	
	Discovery cohort (*n* = 229)	Validation cohort (*n* = 187)
Gender		
Female/male	95/134 (42:58)	78/109 (42:58)
Race		
White/non-white	170/59 (74:26)	0/187 (0:100)
Age (y, median)	52.5 (20-75)	49.0 (17-87)
Previous treatment prior to imatinib		
IFN	98 (43)	30 (16)
Busulfan	17 (7)	0 (0)
Cytarabine	19 (8)	0 (0)
BMT	12 (5)	10 (5)
Newly diagnosed case		
De novo vs. previously treated	115:114	147:40
Disease duration from diagnosis (mo, median)	4.3 (0-231)	0.5 (0-114)
Disease stage		
CP	203 (89)	163 (90)
AP	23 (10)	11 (6)
BC	3 (1)	7 (4)
Cytogenetics		
t(9;22) only	200 (87)	163 (87)
Additional abnormalities	29 (13)*	24 (13)†
Maximum dose of imatinib		
400 mg/d	210 (92)	177 (94)
600 mg/d	17 (7)	7 (4)
800 mg/d	2 (1)	3 (2)

*Additional cytogenetic abnormalities were detected in the discovery population: –Y (*n* = 5); double Ph+ chromosome (*n* = 5); *t*(7;8) (*n* = 2); *t*(9;22;22) (*n* = 2); *t*(2;9;22) (*n* = 2); *t*(9;22;17) (*n* = 1); *t*(1;22;18) with inv(5) (*n* = 1); *t*(7;8) with +8 and +der(22) (*n* = 1); *t*(8;17) (*n* = 1); *t*(8;16) (*n* = 1); inv(9q) (*n* = 1); −18q (*n* = 1); *t*(12;16) (*n* = 1); *t*(3;19) (*n* = 1); *t*(4;6), 47-52, +X, +6, +8, +18, +19, +der(22) (*n* = 1); *t*(17;20), +der(17), +der(20) (*n* = 1); +8 (*n* = 1); –X (*n* = 1).

†Additional cytogenetic abnormalities were detected in the discovery population: –Y (*n* = 3); *t*(7;9;22) (*n* = 1); *t*(8;9;22) (*n* = 1); *t*(9;22;11) (*n* = 1); *t*(9;22;14) (*n* = 1); *t*(9;22;17) (*n* = 1); *t*(9;22;19) (*n* = 1); t (4;22) with *t*(17;20) (*n* = 1); inv(3) (*n* = 1); inv(3) with *t*(12;17), del(7), der(9), add(9), –6, –13 (*n* = 1); der(9), del(9) (*n* = 1); del(7) with *t*(2;11) (*n* = 1); del(22q) (*n* = 1); +der(22) (*n* = 2); +der(22) with *t*(11;13). –18 (*n* = 1); +1 with der(1;15) (*n* = 1); +8 (*n* = 2); +8, +10, +13, +14, +22, der(22) (*n* = 1), +8, +der(22) (*n* = 1); = 12 (*n* = 1).

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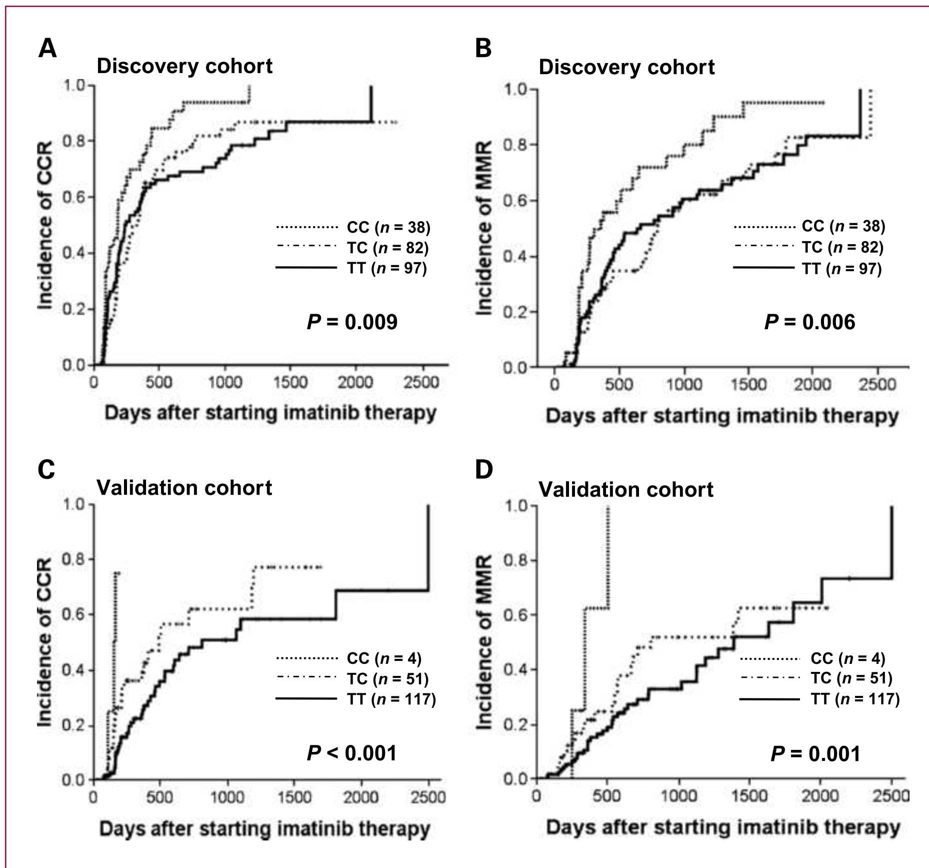

Fig. 1. Differences of CCR and MMR according to the *IFNG* genotype (rs2069705) in the discovery cohort (A and B) and validation cohort (C and D).

and internal validation, correlation of the *IFNG* genotype with CCR and MMR was clearly shown (Table 4), despite of the different frequencies between two cohorts. As shown in Fig. 1 and Table 4, external validation demonstrated that the *IFNG* genotype (rs2069705) was predictive of the probability of CCR (HR, 2.17; $P < 0.0001$; $P_{corr} = 0.002$; Fig. 1C) or MMR (HR, 1.96; $P = 0.0001$; $P_{corr} = 0.005$; Fig. 1C), suggesting that the CC

Table 3. Summary of internal validation using bootstrap survival analysis with all significant SNPs in univariate analyses

Parameter	Gene	SNP ID	HR (95% CI)	Bootstrap, *P* (95% CI)
CCR	*IFNG*	rs1861494	1.289 (1.017-1.634)	0.0356 (1.053-1.550)
	IFNG	rs2069705	1.301 (1.053-1.608)	0.0148 (1.098-1.580)
	CASP9	rs4645981	1.247 (1.011-1.536)	0.0388 (0.676-0.954)
	CASP8	rs3834129	1.247 (1.011-1.536)	0.0388 (0.668-0.947)
	FASL	rs763110	1.357 (1.091-1.689)	0.0061 (0.607-0.876)
	FAS	rs2234767	1.356 (1.032-1.781)	0.0287 (1.097-1.745)
MMR	*IFNG*	rs2069705	1.289 (1.013-1.639)	0.0386 (1.058-1.565)
	FAS	rs2234978	1.291 (0.996-1.672)	0.0532 (1.044-1.553)
	JAK3	rs3212713	1.312 (1.034-1.664)	0.0252 (1.103-1.580)
LOR	*IFNG*	rs2069705	0.607 (0.405-0.910)	0.0157 (0.446-0.822)
	BIRC5	rs9904341	0.578 (0.368-0.907)	0.0172 (0.393-0.808)
TF	*IFNG*	rs2069705	0.630 (0.449-0.883)	0.0074 (0.477-0.819)
	APAF1	rs2288713	0.529 (0.285-0.982)	0.0437 (0.292-0.808)

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Table 4. Result of external validation for the IFNG genotype (rs2069705) in the independent cohort

	***P* value before adjustment**			***P* value after Bonferroni's correction**		
	Additive	**Dominant**	**Recessive**	**Additive**	**Dominant**	**Recessive**
MCR	0.009	0.021	0.016	0.588	1.000	1.000
CCR	<0.0001	0.0067	0.0001	0.002	0.004	0.005
MMR	0.0001	0.0416	<0.0001	0.005	1.000	0.003
LOR	0.513	0.449	0.513	1.000	1.000	1.000
TF	0.273	0.233	0.172	1.000	1.000	1.000

NOTE: Procedures were repeated in additive, dominant, and recessive models with and without Bonferroni's correction. The result showed that the IFNG genotype correlates significantly with the probability of CCR and MMR before adjustment ($P \leq 0.0001$ for CCR, and 0.0001 for MMR) and after Bonferroni's correction ($P = 0.002$ for CCR and 0.005 for MMR) in an additive model.

IFNG genotype (rs2069705) has an approximately 50% higher chance of achieving CCR or MMR compared with the CT/TT genotype.

Rate of response according to the IFNG genotype (rs2069705) in overall patients and in a subgroup of patients with CP CML

To remove the effect of disease status on treatment outcomes, the analysis was repeated in patients confined to those in CP. We compared the rate of CCR at 12 months and of MMR at 18 months according to the *IFNG* genotype (rs2069705) in overall patients and in a subgroup of CML patients in CP (Fig. 2). Significantly higher response rate was noted in favor of the group with the CC genotype both in overall patients (Fig. 2A and B) and in CP patients only (Fig. 2C and D), compared with those with CT or TT genotype. In CP patients, patients with CC genotype showed 76.0% MMR rate at 18 months whereas those with the CT or TT genotype only showed 35.7% and 52.5% MMR rate at 18 months ($P = 0.003$; Fig. 2D).

Multivariate analysis for prediction of cytogenetic and molecular response to imatinib therapy

Multivariate analysis confirmed that the CC *IFNG* genotype (rs2068705) and CP were associated with higher CCR

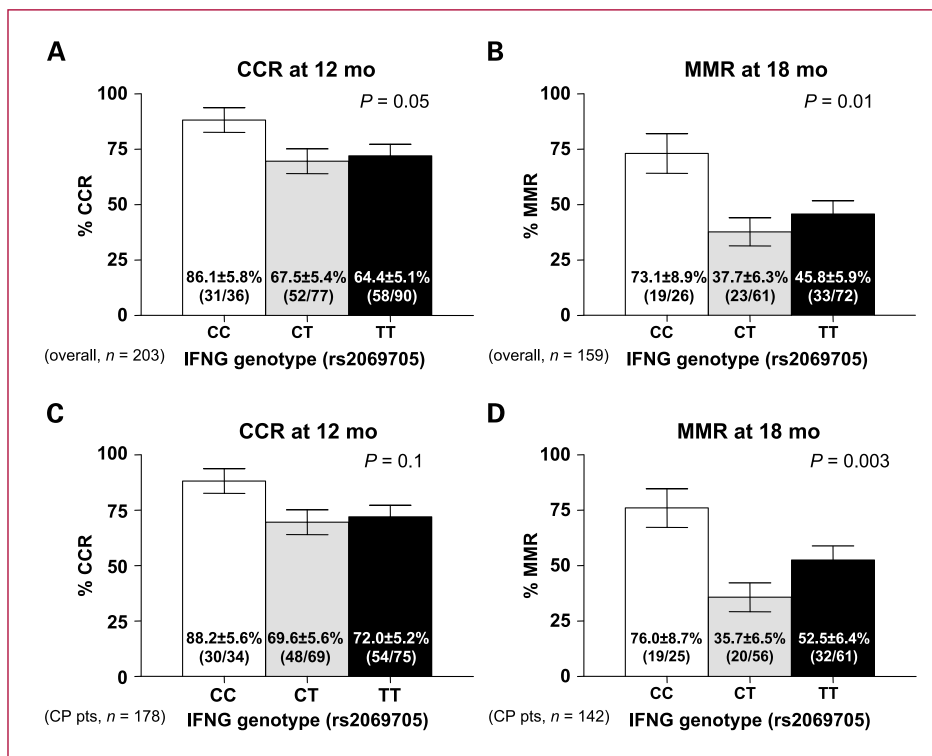

Fig. 2. Response rates according to the *IFNG* genotype (rs2069705). The comparisons in overall patients were presented in A (by CCR at 12 mo) and B (by MMR at 18 mo). In addition, comparisons confined to CP patients were presented in C and D (bars, SE).

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and MMR. With respect to CCR, the patients with CC *IFNG* genotypes showed a higher probability of achieving CCR compared with the CT/TT genotype ($P = 0.005$; HR, 1.727; 1.183-2.519), and those in CP showed higher CCR compared with those in advanced disease stage ($P = 0.023$; HR, 2.024; 1.104-3.704). With regard to MMR, the group with CC *IFNG* genotypes showed significantly better MMR than those with the CT/TT genotype ($P = 0.002$; HR, 1.912; 1.258-2.915).

Discussion

The current study suggested that (*a*) a multiple candidate pathway-based SNP approach could identify several potential predictive markers of response to imatinib therapy; (*b*) among them, the *IFNG* genotype was found to be the significant predictive marker for CCR and MMR, which was confirmed not just by internal validation, but also by external validation using independent external cohort.

Because of the successful introduction of imatinib into CML management, a dramatic improvement in response to imatinib therapy and excellent prognosis have been achieved in CML patients. However, complete eradication of the BCR/ABL-producing clone is obtained in only one quarter of the patients (30). In addition, if imatinib therapy is interrupted after achievement of complete molecular response, a BCR/ABL transcript will reappear and eventually rise up to the diagnostic level in one half of the patients (31). These interindividual variations of the response to imatinib are a practical issue in our daily practice of CML. Thus far, none of the predictive surrogate markers have been successfully applied for the prediction of imatinib response before starting imatinib therapy.

Although the exact mechanism of imatinib resistance is not fully understood, it has been explained with several proposed mechanisms, such as the acquisition of point mutations in the Abl kinase domain, BCR-ABL amplification at the genomic or transcript level (32), overexpression of other tyrosine kinases (i.e., SRC kinase family; ref. 33), variability in the amount and function of the drug influx protein OCT-1, or resistance at the level of "leukemic stem cells" (LSC; ref. 34). Because they are in a quiescent state, LSCs are known to be resistant and insensitive to tyrosine kinase inhibitors, and thus, do not require BCR-ABL signaling for survival (35). Thus, imatinib resistance mechanisms cannot be explained in a simple and singular way, but will be more complex than we speculate. Based on the complexity of the imatinib resistance mechanism, multiple candidate gene SNPs should be examined for their association with imatinib response and resistance.

Of the 79 candidate genotypes in the current study, the *IFNG* genotype was found to be an excellent predictive marker of response to imatinib. The *IFNG* genotype (rs2069705) was consistently confirmed as predictive of achievement of CCR and MMR following imatinib therapy by internal and external validation processes both in Canadian and Korean patients with CML. As shown in Fig. 2, not just in the overall population but also in CP patients, the *IFNG* genotype (rs2069705) was a very predictive marker for CCR at 12 months and MMR at 18 months following imatinib therapy for CML.

Therefore, what is the role of IFN signaling in imatinib therapy for CML? Prior to the introduction of imatinib, IFN-α was widely used as the standard drug for CML patients who were not available for allogeneic transplantation. The exact mechanism of action of IFN-α in the treatment of CML patients has not been fully revealed. A recent study suggested that IFN signaling activates dormant HSCs *in vivo*. Priming of HSCs with IFN-α or chronic IFN-α stimulation followed by 5-fluorouracil treatment was shown for the induction of HSC loss (36). Similar to the action of IFN-α, findings from several investigations have suggested that IFN-γ could promote hematopoiesis and activate the expansion and proliferation of HSCs. IFN-γ promotes *ex vivo* expansion of the earliest CD34+ hematopoietic precursors (37), and thus, stimulates the early stage of myelopoiesis in the presence of certain growth factors (38). In addition, it induces *in vivo* and *in vitro* expansion of $Lin^{neg}Sca1^{+}c\text{-}kit^{+}$ progenitor cells (39). Kurz and colleagues reported that IFN-γ stimulates CD34+ cells, which could induce tryptophan degradation and neopterin formation (40). This might explain the induced proliferation of HSCs and LSCs by IFN-γ, which results in sensitization of CML LSCs to imatinib, as myeloid cytokines (i.e., granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor) decrease quiescent CML LSCs and promote their elimination by imatinib (41, 42).

The *IFNG* gene is located on chromosome 12. The SNP of rs2069705 is a regulatory sequence affecting IFN-γ expression as a promoter. In the present study, the level of mRNA or protein of IFN-γ, according to the *IFNG* genotype (rs2069705), was not assessed. Thus, further studies will be needed to evaluate the implications of the CC/CT/TT genotype on the expression level of IFN-γ, although other studies suggested a strong association between the *IFNG* genotype (rs2069705) and a susceptibility to systemic lupus erythematosus (43), which implies a strong association between the *IFNG* genotype and the IFN-γ expression levels.

Not just in imatinib therapy, but also in nilotinib therapy, IFN signaling can be involved in the mechanism of action of TKI therapy. A previous study revealed that nilotinib inhibits the proliferation and function of CD8+ T-lymphocytes through T-cell receptor signaling, thus resulting in a decreased release of IFN-γ (44). In addition, the inhibitory effect of nilotinib was reported to be two times stronger compared with imatinib. Accordingly, in future studies, it might be interesting to look at the effect of *IFNG* SNPs in nilotinib therapy for CML.

In conclusion, findings from the current study suggest that (*a*) a multiple candidate pathway-based SNP approach can identify several potential predictive markers of response to imatinib therapy; (*b*) among them, the *IFNG* genotype was found to be the most significant SNP marker for the prediction of CCR and MMR, which was confirmed not just by internal validation, but also

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by external validation in the independent external cohort. This finding proposes the potential involvement of the IFN signaling pathway in the mechanism of action of imatinib, particularly in CML LSCs. Further detailed genetic and functional studies of *IFNG* genes will be helpful to reach a clear conclusion on the role of the *IFNG* gene in the mechanism of action of imatinib therapy in CML.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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