ABL single nucleotide polymorphisms may masquerade as BCR-ABL mutations associated with resistance to tyrosine kinase inhibitors in patients with chronic myeloid leukemia

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ABSTRACT

The BCR-ABL K247R change is based on a rare single nucleotide polymorphism occurring likewise in healthy controls and non-hematologic cell types. Despite its juxtaposition to the P-loop, functional analysis showed no alteration compared to non-mutated *BCR-ABL*. We sought to investigate if other changes in the *BCR-ABL* kinase domain should be considered as single nucleotide polymorphisms rather than acquired mutations. A total of 911 chronic myeloid leukemia patients after failure or suboptimal response to imatinib were screened for *BCR-ABL* kinase domain mutations. Single nucleotide polymorphism analysis was based on the search for nucleotide changes in corresponding normal, non-translocated ABL alleles by ABL allele-specific PCR following mutation analysis. In addition to the K247R polymorphism we uncovered five new single nucleotide polymorphisms within the *BCR-ABL* kinase domain; two of them led to amino acid changes. Single nucleotide polymorphisms could theoretically contribute to primary but not to secondary resistance to tyrosine kinase inhibitors and must therefore be distinguished from acquired mutations. Novel point mutations should be confirmed by analyzing the normal ABL alleles to exclude polymorphisms.

Key words: single nucleotide polymorphism, polymorphism, *BCR-ABL* mutation, imatinib resistance, chronic myeloid leukemia.

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Introduction

In comparison to acquired mutations, single nucleotide polymorphisms (SNPs) are evolutionarily inherited point mutations representing the most common form of genetic variation in the human genome.[1] About three million SNPs have been characterized by the International HapMap Project so far.[2] It is estimated that over ten million SNPs exist throughout the genome, at an average of one SNP every 300-1000 base pairs.[1] SNPs contribute to wide variations in how individuals respond to medication, either by changing pharmacokinetics or by altering the cellular response.[3] In CML, polymorphic variations have been described, for example, in the TP53 gene associated with response to imatinib treatment and at the STAT5 locus in association with therapy with interferon α.[4,5]

In the *BCR-ABL* fusion gene, the amino acid change K247R is based on a rare adenine to guanine SNP at position 58778 (GenBank accession N. U07563) within the ABL kinase domain.[6,7] Despite its juxtaposition to the P-loop, biochemical and cellular assays of imatinib and dasatinib sensitivity showed no significant alteration compared to non-mutated *BCR-ABL*.[8] This indicates that polymorphisms within the BCR-ABL kinase domain do not necessarily imply a change of treatment – unless there are signs of inadequate response to treatment – and therefore must be distinguished from acquired mutations leading to resistance. International recommendations suggest the confirmation of newly identified mutations by amplification of the normal ABL alleles to exclude polymorphisms.[9]

In this study, we systematically analyzed whether some changes in the BCR-ABL kinase domain should be considered as SNPs rather than acquired mutations. In comparison to acquired *BCR-ABL* mutations, SNPs also have to occur in nor-

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mal, non-translocated ABL alleles. ABL allele-specific PCR was performed for exclusive amplification of normal ABL alleles followed by mutational analysis of the entire ABL kinase domain.

Design and Methods

Patients' samples

Peripheral blood samples from 911 CML patients (501 male, 410 female; median age 58.1 years, range 15.5-85.3) with failure or suboptimal response to imatinib according to the European LeukemiaNet recommendations[10] were investigated after written informed consent was obtained. Patients were in chronic phase (n=587), accelerated phase (n=151), myeloid (n=103), or lymphoid blast crisis/ALL (n=70). All patients had previously received imatinib between 400 and 800 mg/day. The clinical trials were conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000, and approved by national and/or international ethics committees.

RNA extraction and cDNA synthesis

Total RNA was extracted after hypotonic red cell lysis from at least 20 mL of peripheral blood using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) or the TRIzol® reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. cDNA synthesis was performed using random hexamer primers and MMLV reverse transcriptase (Invitrogen) as previously described.[11]

D-HPLC analysis

Screening for kinase domain mutations of translocated BCR-ABL alleles was performed by denaturing high-performance liquid chromatography (D-HPLC) on a Transgenomic Wave® System (Transgenomic, Omaha, NE, USA) and direct sequencing according to previous descriptions.[12] To test whether these mutations also occur in corresponding normal, non-translocated ABL alleles, ABL allele-specific PCR was performed. In the first round of amplification the following primers were used: ABL-1a for (5'-CTGGTGGGCTGCAAATCCAAGAA-3'), ABL-1b for (5'-TACTTGGGGACCAAAGAAGG-3'), and ABL-A rev (5'-ATGGTCCAGAGGATCGCTCTCT-3'). In the second round of amplification the coding sequence of the ABL kinase domain was divided into three partially overlapping fragments, ABL-B (codons 207-324, 401bp), ABL-C (codons 279-414, 457bp), and ABL-D (codons 382-517, 453bp). One µL of 1:100 diluted first step PCR products was used as template for the second step PCR. PCR products were analyzed by D-HPLC. PCR and D-HPLC conditions were identical to previous descriptions for mutation analysis of translocated BCR-ABL alleles.[12]

Direct sequencing

Direct sequencing was performed in both directions for all samples with suspect D-HPLC profiles on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were compared with the ABL wild-type sequence (NCBI GenBank accession N. U07563 for the ABL 1a and U07561 for the ABL 1b splice variant respectively).

Cloning

For confirmation of nucleotide changes in normal ABL alleles, ABL-A fragments amplified by ABL-1a for and ABL-A rev primers were cloned into a pCR2.1-TOPO-vector using the TOPO® TA Cloning Kit (Invitrogen) according to the manufacturer's protocol. Each ten individual clones were selected, amplified and sequenced as described above.

Allele frequencies

The calculation of SNP allele frequencies is based on the observance of BCR-ABL kinase domain mutation analysis corresponding to an investigation of 911 different BCR-ABL alleles. Allele frequencies were calculated using the following ratio: total number of patients with the respective polymorphism in BCR-ABL alleles/911 total BCR-ABL alleles.

Results and Discussion

BCR-ABL kinase domain mutations were detected in 456/911 patients (50%). Eighty-three different BCR-ABL mutations affecting 60 amino acids were revealed (Table 1). In the current literature, the number of different BCR-ABL kinase domain mutations described in patients thereby exceeds 100, affecting more than 70 amino acids.[13]

ABL allele-specific PCR amplification of corresponding normal, non-translocated ABL alleles and subsequent mutation analysis was performed for all 83 different types of BCR-ABL kinase domain mutations. Depending on the availability of samples, 41 mutations were analyzed in two different patients and 42 mutations in one patient respectively. In 116/124 patients (94%) the mutation analysis of normal ABL alleles showed wild-type profiles indicating acquired mutations in corresponding BCR-ABL alleles. In 8 patients (6%) the mutation analysis revealed nucleotide changes within the kinase domain of normal ABL alleles indicating SNPs. Detailed clinical information of these patients is given in the *Online Supplementary Table*. Identical mutations were detected as initially found in translocated BCR-ABL alleles: 58758 G/A (T240T), 58778 A/G (K247R; n=2), 68708 T/G (F311V), 68722 T/G (T315T), 68736 A/G (Y320C), and 74901 A/G (E499E; n=2) (Figure 1). Sequencing patterns of normal ABL alleles showed both mutant and wild-type clones. In BCR-ABL alleles, either mutant or wild-type clones were observed in patients whose SNP or wild-type carrying ABL allele presumably translocated to BCR-ABL. In all 8 patients the nucleotide changes were confirmed by cloning experiments to be located in normal, non-translocated ABL alleles. Interestingly, a second patient harboring the substitution 68708 T/G (F311V) in BCR-ABL alleles did not show a nucleotide change in normal ABL alleles indicating that polymorphisms could behave as acquired mutations. Allele frequencies of SNPs within the BCR-ABL kinase domain are given in Table 2 and were calculated on the basis of mutation analysis from 911 CML patients. The 74901 G (E499E) change was the

most frequently observed polymorphism with an allele frequency of 73/911 (8.0%). In this cohort of patients, the previously described 58778 G (K247R) polymorphisms had a frequency of 1.0%, whereas the nucleotide changes 58758 A, 68722 G, and 68736 G were detected once only leading to an allele frequency of 0.1% each. The traditional definition of SNP sets the minimal allele frequency at 1% or greater in at least one population.[14] Therefore, it is not yet clear whether these nucleotide changes should be referred to as SNPs or rather as rare single nucleotide variants.

Within the ABL kinase domain, the following SNPs are listed in major SNP databases (NCBI, ENSEMBL) so far: rs2229069 (T240T), rs34549764 (K247R), rs1141212 (L298L), rs1141213 (L354L), rs1064156 (E459K), rs2227985 (E499E). Three of them (rs2229069, rs34549764, rs2227985) were also detected in our cohort of patients. However, we could not confirm the E459K substitution as a polymorphism since 2 investigated patients with 73279 G/A (E459K) BCR-ABL alterations showed wild-type ABL alleles. This discrepancy could also be explained by the possibility that polymorphisms could behave like mutations as discussed above for the F311V change. In addition to the SNPs listed in databases, we found the following: 68708 T/G (F311V), 68722 T/G (T315T), and 68736 A/G (Y320C). The clinical impact of these SNPs is not yet known and needs further investigation. Albeit only 2 of our detected SNPs led to amino acid changes, even polymorphisms which do not lead to amino acid changes (silent mutations) can affect protein expression and function by altering mRNA stability.[15,16]

Table 1. Analyzed BCR-ABL mutations and polymorphisms.

Nucleotide position[1]	*Nucleotide change*	*Amino acid change*[2]	*N*[3]	*Nucleotide position*[1]	*Nucleotide change*	*Amino acid change*[2]	*N*[3]
58687	G/C	A217P	1	68801	T/C	Y342H	1
58732	T/C	Y232H	1	68808	C/T	A344V	2
58758	G/A	T240T	1	68826	C/T	A350V	1
58763	T/C	I242T	1	68828	A/G	M351V	2
58768	A/G	M244V	2	68829	T/C	M351T	2
58776	C/T	H246H	1	68841	A/C	E355A	1
58778	A/G	K247R	2	68841	A/G	E355G	2
58780	C/G	L248V	2	68852	T/G	F359V	2
58787	G/A	G250E	2	68852	T/A	F359I	2
58787	G/T	G250V	1	68853	T/G	F359C	2
58794	G/T	Q252H	2	70697	C/A	L364I	2
58795	T/C	Y253H	2	70701	C/T	A365V	1
58796	A/T	Y253F	2	70716	T/C	L370P	1
58801	G/A	E255K	2	70730	C/T	H375Y	1
58802	A/T	E255V	2	70741	G/A	K378K	1
58812	G/T	E258D	1	70742	G/A	V379I	2
58820	G/T	W261L	1	70757	C/A	L384M	2
67954	G/A	E275K	1	70766	T/A	L387M	2
67954	G/C	E275Q	1	70766	T/G	L387V	1
67958	A/G	D276G	2	70768	G/T	L387F	2
67960	A/G	T277A	1	70769	A/T	M388L	2
67966	G/A	E279K	2	70794	A/C	H396P	2
67970	T/C	V280A	1	70794	A/G	H396R	2
67972	G/T	E281X	1	70796	G/C	A397P	2
67986	A/G	K285K	1	70857	C/T	S417F	1
67998	C/T	V289V	1	70857	C/A	S417Y	1
68005	G/C	E292Q	1	70859	A/G	I418V	1
68006	A/T	E292V	1	70860	T/G	I418S	1
68008	A/G	I293V	1	73226	C/T	P441L	2
68023	C/G	L298V	1	73252	G/A	E450K	1
68026	G/T	V299L	2	73253	A/C	E450A	2
68708	T/A	F311I	2	73253	A/G	E450G	2
68708	T/G	F311V	2	73261	G/A	E453K	2
68709	T/A	F311Y	1	73262	A/T	E453V	1
68710	C/A	F311L	2	73279	G/A	E459K	2
68720	A/G	T315A	1	73320	G/A	M472I	1
68721	C/T	T315I	2	74861	T/C	F486S	2
68722	T/G	T315T	1	74901	A/G	E499E	2
68726	T/A	F317I	1	74916	C/T	D504D	1
68728	C/A	F317L	2	75266	A/G	E507G	1
68736	A/G	Y320C	1	75286	G/A	G514S	1
68748	T/A	L324Q	2				

[1]Nucleotide positions according to GenBank accession number U07563 for the ABL 1a splice variant. [2]Amino acid residues are denoted with the single letter code and correspond to the ABL 1a variant. [3]Total number of patients with the respective BCR-ABL mutation of the corresponding normal, untranslocated ABL alleles were investigated.

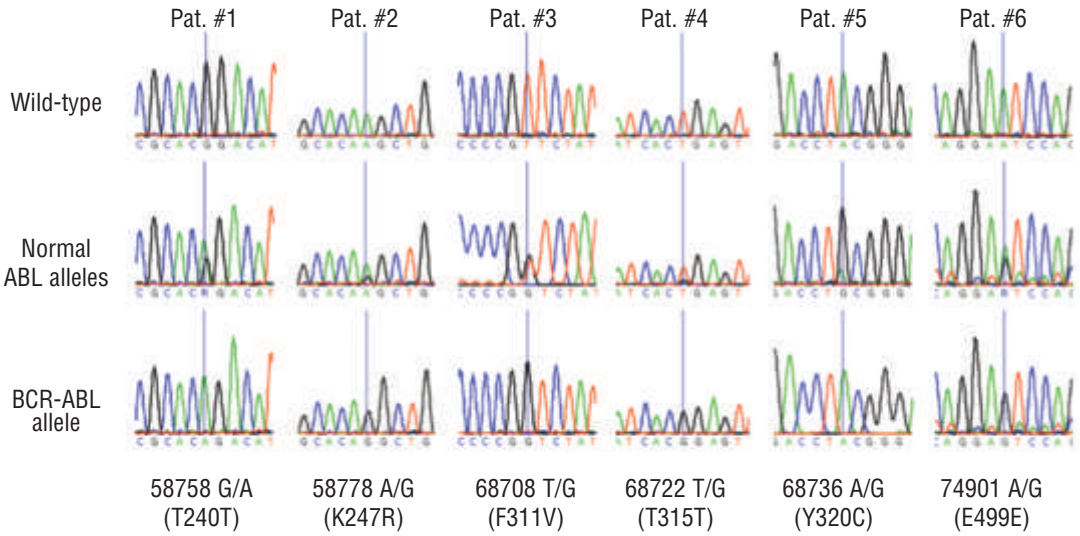

Figure 1. Sequencing analysis of corresponding wild-type, normal ABL and translocated BCR-ABL alleles in 6 patients harboring single nucleotide polymorphisms (SNPs). Nucleotides of interest are marked by lines. Nucleotide positions (GenBank accession N. U07563), nucleotide changes and corresponding amino acid changes are indicated below. Both mutant and wild-type clones were apparent in normal ABL alleles whereas either mutant or wild-type clones were observed in translocated BCR-ABL alleles.

Concerning the pathogenetic relevance of BCR-ABL mutations associated with imatinib resistance, our data show that mutations do not pre-exist on normal ABL alleles before translocation to BCR-ABL. In total, 80 clones spanning normal ABL alleles were amplified and sequenced without detection of any known amino acid substitution leading to imatinib resistance. The following observations suggest that BCR-ABL kinase domain mutations may be part of the natural disease evolution. The frequency of BCR-ABL mutations is higher in advanced CML phases and increases with disease duration.[17,18] Mutation specific PCR of pre-therapeutic samples revealed the same mutation type as detected at relapse, consistent with selection of resistant clones during therapy.[19,20] However, it is important to note that BCR-ABL mutated clones do not always seem to be selected in the same way or explain clinical resistance to imatinib.[21,22]

The *T315I* mutation is considered to be of particular importance due to its complete resistance even to second generation tyrosine kinase inhibitors.[23] Screening for this mutation is frequently performed by mutation-specific PCR assays. Our detection of the polymorphism 68722 T/G (T315T) is of special interest because these methods could falsely recognize this nucleotide change as a T315I mutation. We found this polymorphic variation in a 45-year-old male chronic phase CML patient who has shown a good molecular response on 600mg imatinib for 37 months. Although the SNP seems to be very rare, investigators should keep its existence in mind.

To summarize, the vast majority of BCR-ABL kinase domain mutations are acquired mutations rather than SNPs. However, polymorphisms must be distinguished from acquired mutations because they cannot contribute to secondary resistance to tyrosine kinase inhibitors. Analysis of normal ABL alleles enables an easy and fast differentiation between SNPs and acquired mutations. Pre-existing polymorphisms not representing acquired mutations do not directly require a change in therapeutic strategy, unless there are signs of an inadequate response to treatment.

Table 2. Allele frequencies of SNPs within the BCR-ABL kinase domain in 911 analyzed chronic myeloid leukemia patients.

Nucleotide position[1]	*Nucleotide polymorphism*	*Amino acid change*[2]	*N*[3]	*Allele frequency (%)*
58758	A	T240T	1	0.1
58778	G	K247R	9	1.0
68708	G	F311V	2	0.2
68722	G	T315T	1	0.1
68736	G	Y320C	1	0.1
74901	G	E499E	73	8.0

[1]Nucleotide positions according to GenBank accession number U07563 for the ABL 1a splice variant. [2]Amino acid residues are denoted with the single letter code and correspond to the ABL 1a variant. [3]Total number of patients harboring the respective polymorphism.

Authorship and Disclosures

TE: principal investigator, designed and performed the research, analyzed the data and wrote the manuscript; JH, PE, BH, AL, RH: contributed to the design of the study, data collection and interpretation of results; AH, MM: designed the research and wrote the manuscript.

The authors reported no potential conflicts of interest

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