Dynamics of mutant *BCR-ABL*-positive clones after cessation of tyrosine kinase inhibitor therapy

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ABSTRACT

Background

Point mutations of the *BCR-ABL* tyrosine kinase domain are considered the predominant cause of imatinib resistance in chronic myeloid leukemia. The expansion of mutant *BCR-ABL*-positive clones under selective pressure of tyrosine kinase inhibition is referred to as clonal selection; there are few data on the reversibility of this phenomenon.

Design and Methods

The changes of expression of mutant *BCR-ABL*-positive alleles after cessation of tyrosine kinase inhibitor treatment were examined in 19 patients with chronic myeloid leukemia harboring different mutations in a longitudinal follow-up. The proportion of mutant alleles was quantified by amplification of rearranged *ABL* sequences followed by mutation-specific restriction digestion, electrophoresis and densitometry. The size of mutant clones was established as a measure of the absolute amount of mutant cells considering the proportion of mutant *BCR-ABL* transcripts and the total level of *BCR-ABL* obtained by quantitative reverse transcriptase polymerase chain reaction.

Results

The median proportion of mutant transcripts was 97% before and 8% after cessation of tyrosine kinase inhibitor treatment indicating a relative decline of 88% within a median of 6 months. The relative decrease in the size of the mutant clones was 86%. Repeated selection and deselection of the mutant clone after resumption and second cessation of tyrosine kinase inhibitor treatment was observed in individual patients.

Conclusions

Deselection of mutant *BCR-ABL*-positive clones after cessation of tyrosine kinase inhibitor treatment might be a common, rapid and reproducible phenomenon, although some patients harboring the T315I mutation showed no deselection. Cessation of tyrosine kinase inhibitor treatment may lead to the regression of T315I mutant clones to a level under the limit of detection, offering the therapeutic option of resumed tyrosine kinase inhibitor treatment under close surveillance of the mutation status.

Key words: chronic myeloid leukemia, imatinib resistance, drug resistance, *BCR-ABL* mutation.

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Introduction

Point mutations of the *BCR-ABL* tyrosine kinase domain are the major cause of imatinib resistance in chronic myeloid leukemia (CML).[1-3] Up to now more than 100 mutations have been described affecting more than 70 amino acids causing resistance by heterogeneous molecular mechanisms.[4-6] Highly sensitive methods for the detection of mutations in the case of suboptimal response have been introduced in the management of CML.[1,7,8] Mutant BCR-ABL-positive clones have been shown to pre-exist treatment in imatinib-naïve patients and to outgrow the unmutated clone under the selective pressure of tyrosine kinase inhibition in a process known as clonal selection.[9-12] Anecdotal cases of reversibility of clonal selection after cessation of treatment with a tyrosine kinase inhibitor (TKI) have been reported.[13-15]

In theory, the proliferative advantage of a resistant clone in the presence of a TKI does not imply a proliferative disadvantage in the absence of the TKI, indicating that the general assumption of clonal deselection after TKI discontinuation remains controversial. Besides, inhibition of drug affinity mutations may also contribute to alterations in the activity and substrate specificity of the BCR-ABL tyrosine kinase suggesting a possible gain or loss of function.[2,16-19] Furthermore, clinical studies have revealed mixed dynamics in the outgrowth of mutant clones in the presence of imatinib supporting the notion that the model of clonal selection alone does not fully reflect the *in vivo* situation.[10,20,21] Recently, it has been reported that imatinib-resistant mutant clones can disappear in patients receiving treatment with second-generation TKI.[22,23]

In this study we sought to investigate the dynamics of mutant *BCR-ABL* alleles in 19 CML patients resistant to imatinib and exposed to alternative non-TKI treatment modalities.

Design and Methods

Patients

Between 2001 and 2007, 75 CML patients who had hematologic resistance to TKI therapy due to a *BCR-ABL* kinase domain mutation detected by direct sequencing were identified (44 males, 31 females; median age 62 years, range 30-80). The disease phase at the start of TKI treatment was chronic phase (n=35), accelerated phase (n=25), and blast crisis (n=15). All patients were initially treated with imatinib; one patient was switched to nilotinib and one to dasatinib after failure of imatinib therapy.

A subgroup of 19 patients (11 males, 8 females; median age 63 years, range 31-73) received subsequent alternative treatment consisting of non-specific chemotherapy (17 after imatinib, one after imatinib/nilotinib, one after imatinib and subsequently after dasatinib, patient n. 8, Table 1), and had a follow up of at least 4 months after the change of treatment regimen (median 13 months, range 4-33). The disease phase at the onset of TKI treatment was

Table 1. Patient and treatment characteristics.

Patient N.	Age, sex	*BCR-ABL* mutation	Time from diagnosis to start of TKI (years)	Therapy prior to TKI	Disease phase at the onset of TKI	TKI	Time from onset of TKI to detection of mutation (months)	Duration of TKI treatment (months)	Chemotherapy after cessation of TKI under follow up of mutant clone
1	63, F	Y253F	12.6	HU, Busulfan	BC	Imatinib	43.9	47.1	HU, ARA-C
2	52, F	Y253H	6.4	HU, IFN, ARA-C, Anagrelide	CP	Imatinib	25.3	32.5	HU, Anagrelide
3	72, M	Y253H	2.7	HU, IFN, Busulfan	AP	Imatinib	15.6	17.1	HU, ARA-C
4	60, F	Y253H	25.7	CCNU, Busulfan, ARA-C, HU, IFN	CP	Imatinib	8.5	9.3	HU, Mercaptopurine
5	62, F	Y253H	0.1	HU	BC	Imatinib	22.9	24.6	HU, Mercaptopurine, Idarubicin, ARA-C
6	65, F	E255K	2.1	HU, IFN	CP	Imatinib	5.0	3.1	HU
7	44, M	E255K	0.8	HU, IFN, ARA-C	CP	Imatinib	15.9	18.8	HU, ARA-C
8 (1)	66, F	E255K	6.1	HU, IFN, ARA-C, Anagrelide	CP	Imatinib	20.3	28.5	ARA-C, Anagrelide, HU, Omacetaxine
8 (2)	69, F	T315I	9.2	ARA-C, Anagrelide, HU, Omacetaxine	AP	Dasatinib	21.0	19.0	HU
9	35, M	E255K	5.1	HU, IFN	CP	Imatinib	25.5	47.0	HU
10	72, M	E255K	3.6	HU	BC	Imatinib	29.4	29.4	HU
11	54, M	T315I	6.9	HU, ARA-C, Busulfan	AP	Imatinib	11.9	13.8	ARA-C
12	63, F	T315I	1.3	HU, IFN	CP	Imatinib	14.3	15.2	Mercaptopurine, Anagrelide, IFN, HU
13	73, M	T315I	3.4	HU	AP	Imatinib	8.8	8.8	Thioguanine
14	70, M	T315I	0.3	HU	CP	Imatinib	18.3	22.4	IFN
15	70, F	T315I	0.0	None	CP	Imatinib	10.3	11.0	HU
16	58, M	T315I	4.1	HU, IFN, ARA-C, Anagrelide	CP	Imatinib, Nilotinib	57.2	75.6	HU
17	31, M	T315I	0.1	HU	CP	Imatinib	6.0	6.0	HU
18	57, M	M351T	5.3	HU, IFN	BC	Imatinib	17.3	41.0	Busulfan
19	70, M	M351T	1.3	HU, IFN, ARA-C, Anagrelide	AP	Imatinib	18.4	31.5	HU, ARA-C, Anagrelide

HU: hydroxyurea; ARA-C: cytarabine; IFN: interferon α; BC: blast crisis; CP: chronic phase; AP: accelerated phase.

chronic phase in 11 patients, accelerated phase in five patients and blast crisis in four (patient n. 8 started imatinib treatment in chronic phase and dasatinib treatment in accelerated phase). This subgroup expressed five different mutations, for which a semiquantitative detection assay had been established: Y253F, n=1; Y253H, n=4; E255K, n=5; T315I, n=8; M351T, n=2 (Table 1). Patient n. 8 was examined for two different mutations at two time-points: the first mutation (E255K) emerged under imatinib treatment and disappeared after its cessation, the second (T315I) occurred under dasatinib treatment. Patient n. 16 showed the D276G mutation under imatinib; this mutation was lost after switching to nilotinib therapy. Subsequently, the T315I mutation emerged which was retrospectively found to have been present in a small clone during the prior imatinib therapy. All patients were included in studies approved by the Heidelberg University Institutional Review Board and gave informed consent to participation in this study according to the Declaration of Helsinki.

Real-time quantitative polymerase chain reaction, direct sequencing

RNA was extracted from total peripheral blood leukocytes and reverse transcribed according to standard methods.[24] Real-time quantitative polymerase chain reaction (PCR) was performed to determine the numbers of transcripts of *BCR-ABL* and the control genes *ABL* and β-glucuronidase (*GUS*).[25,26] A 675 bp product encoding *ABL* amino acids 207 to 414 was generated using a hemi-nested PCR to allow specific amplification of the translocated *ABL* alleles.[3] Products were directly sequenced in both directions and compared with the wild-type *ABL* sequence (GenBank accession # U07563).

Quantification of the proportion of mutant BCR-ABL alleles

A mutation-specific nested PCR generated transcripts flanking the different mutation sites. The first step used the primers B2B (BCR exon 13) ACAGCATTCCGCTGACCATCAATAAG and A7– (ABL exon 7) AGACGTCGGACTTGATGGAGAACT spanning the *BCR-ABL* junction site, thus ensuring that the normal unrearranged *ABL* allele was not amplified and subjected to analysis. The second step comprised mutation-specific primers yielding products of different lengths. Mutation-specific restriction digestion was performed to quantify the proportion of mutant alleles. Mutations led to loss of *Rsa*I (codon 253), *Mnl*I (codon 255), *Dde*I (codon 315), and *Nco*I (codon 351) restriction sites. Gel electrophoresis on 1.8% ethidium bromide-stained agarose gels showed the proportion of mutant alleles by differential band intensity of the digested and the undigested transcripts. A correction factor was used to compensate for differences of band intensity due to different transcript lengths (Table 2, Figure 1). The linearity of the assay was demonstrated using dilutions of pCR®2.1-TOPO® plasmids (Invitrogen, Carlsbad, CA, USA) harboring mutations in unmutated pCR®2.1-TOPO® plasmids.[3]

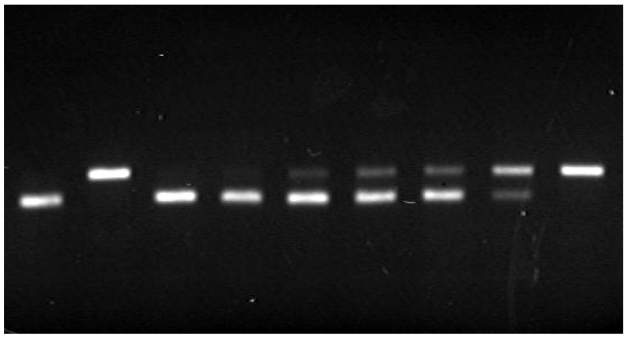

Figure 1. Restriction digest assay for a dilution of Y253H mutant *BCR-ABL* in mutation-negative *BCR-ABL* (tracks 3-9). The lower band represents the long fragment after digestion of mutation-negative transcripts, the upper band the undigested, i.e. the mutant transcripts. Mutation-negative and mutant controls (tracks 1 and 2).

Results

Proportion of mutant BCR-ABL transcripts

The proportion of mutant *BCR-ABL* transcripts is given by the ratio of mutant *BCR-ABL* transcripts to total *BCR-ABL* transcripts ($BCR\text{-}ABL^{mut}/BCR\text{-}ABL^{total}$). Assuming a homogeneous transcriptional activity of mutated and unmutated *BCR-ABL*-positive cells, the proportion of mutant *BCR-ABL* transcripts reflects the proportion of mutant *BCR-ABL*-positive alleles and cells in total *BCR-ABL*-positive alleles and cells.

Overall, the median proportion of mutant transcripts was 97% (range, 38-100%) before cessation of TKI treatment and 8% after cessation of TKI treatment (range, 0-100%; $P<0.0001$) indicating a relative decline of 88% (range, 13-100%; Table 3). The median time to the nadir (lowest proportion) of mutant alleles after cessation of TKI treatment was 6 months (range, 1-26 months; Table 3).

Mutations showed different patterns of deselection (Figure 2A). The P-loop mutations examined (Y253F/H and E255K) showed an almost complete regression of mutant transcripts under non-specific chemotherapy. In

Table 2. PCR primers and digestion products. Lower case letters indicate mismatches introduced to avoid further restriction sites.

Mutation	Forward primer	Reverse primer	Restriction enzyme	Size of the digested unmutated fragments (bp)	Undigested mutant fragment (bp)
Y253F/H	A4+ (ABL exon 4) TCACCACGCTCCATTATCCA	RSA- (ABL exon 4) TCTTCCACACGCCCTCaTAaACCT	*Rsa*I	138 + 30	168
E255K	A4+ (ABL exon 4) TCACCACGCTCCATTATCCA	A4- (ABL exon 4) CTTCCACACGCtCTCGTACA	*Mnl*I	136 + 31	167
T315I	A4/5+ (ABL exon 4/5) AAGACCTTGAAGGAGGACACCATG	A6- (ABL exon 6) GTTGCACTCCaTCAaGTAGTCCA	*Dde*I	134 + 49	183
M351T	A6+ (ABL exon 6) CCCGTTCTATATCATCACTGAGTTC	A7- (ABL exon 7) AGACGTCGGACTTGATGGAGAACT	*Nco*I	218 + 123	341

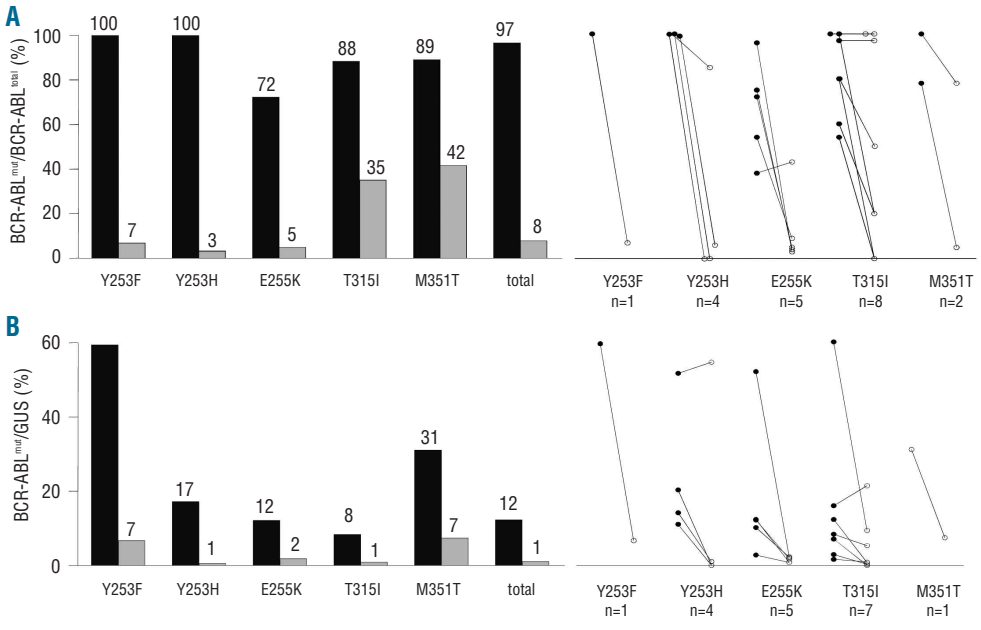

Figure 2. (A) Median proportion of mutant transcripts before (black columns) and after (gray columns) cessation of TKI treatment for different mutations (left). Proportions of mutant transcripts for single patients before (left dots) and after (right dots) cessation of TKI treatment (right). (B) Median size of mutant clones before (black columns) and after (gray columns) cessation of TKI treatment for different mutations (left). Sizes of mutant clones for single patients before (left dots) and after (right dots) cessation of TKI treatment (right).

Table 3. Proportion (light gray columns) and size (dark gray columns) of mutant clone before and after cessation of TKI treatment, time to lowest proportion for single patients.

Patient N.	Under TKI treatment				After cessation of TKI treatment				Time from cessation of TKI treatment to lowest proportion of mutant *BCR-ABL* (months)	Relative decline of the proportion of mutant transcripts (%)	Relative decline of the size of the mutant clone (%)
	BCR-ABL (mutated)/ *BCR-ABL* (total) (%, proportion)	*BCR-ABL* (total) /*ABL* (%)	*BCR-ABL* (total) /*GUS* (%)	*BCR-ABL* (mutated) /*GUS* (%, size)	*BCR-ABL* (mutated)/ *BCR-ABL* (total) (%, proportion)	*BCR-ABL* (total) /*ABL* (%)	*BCR-ABL* (total) /*GUS* (%)	*BCR-ABL* (mutated) /*GUS* (%, size)			
1	100	64	59.5	59.5	7	38	26.3	6.7	26	93	89
2	100	50	11.0	11.0	0	81	6.9	0.0	7	100	100
3	100	61	14.1	14.1	6	30	7.7	1.0	19	94	93
4	100	100	20.3	20.3	0	97	37.2	0.0	5	100	100
5	99	100	52.4	51.6	85	100	64.0	54.6	2	13	-6
6	96	100	54.0	52.0	3	90	70.0	2.1	2	97	96
7	72	100	16.8	12.2	5	78	36.5	1.8	7	93	85
8 (1)	38	18	7.3	2.8	43	8	1.8	0.8	2	-13	71
8 (2)	80	9	2.0	1.6	50	3	1.7	0.9	4	38	44
9	75	66	13.6	10.2	4	100	64.5	2.3	2	95	77
10	54	93	22.9	12.3	9	31	8.5	0.8	1	83	93
11	97	100	63.3	60.3	97	44	9.7	9.4	11	0	84
12	100	51	16.0	16.0	100	30	21.4	21.4	4	0	-34
13	100	100	8.4	8.4	100	15	5.3	5.3	9	0	37
14	54	15	13.0	7.1	0	100	15.6	0.0	6	100	100
15	100	10	2.9	2.9	20	21	1.5	0.3	2	80	90
16	60	33	20.6	12.3	20	13	3.9	0.8	7	67	93
17	80	48	ND	ND	0	32	11.3	0.0	6	100	ND
18	78	30	35.7	1.8	5	62	ND	ND	24	94	ND
19	100	76	31.1	31.1	78	97	9.5	7.4	3	22	76
Median	96.5	62.5	16.8	12.3	8.0	41.0	9.7	1.0	5.5	88	87.0

The proportion of mutant transcripts is given by the ratio of mutant BCR-ABL *transcripts to total BCR-ABL transcripts (light gray columns). The size of mutant clones is defined by the number of mutant* BCR-ABL *transcripts divided by the number of transcripts of the control gene* GUS, *thus reflecting the absolute number of mutant* BCR-ABL *positive cells (dark gray columns). The number of mutant* BCR-ABL *positive transcripts was calculated by multiplying the proportion of mutant transcripts by the number of total* BCR-ABL *transcripts.* BCR-ABL/ABL *and* BCR-ABL/GUS *ratios can be converted to* BCR-ABL *(IS) using conversion factors according to the international scale (IS). (ND, not determined)*

contrast, the median expression of mutant transcripts remained at higher levels for the T315I and M351T mutations. The patterns for T315I were heterogeneous: mutant transcripts declined under the level of threshold in two patients (patients # 14 and 17), whereas three other patients expressed 100% mutant *BCR-ABL* until the end of follow-up (patients n. 11-13). The median relative decline of the proportion of mutant *BCR-ABL* transcripts ranged from 52% to 97% for the different subgroups of mutations (Table 4).

Size of mutant clones

While the proportion of mutant *BCR-ABL*-positive transcripts reflects the proportion of mutant *BCR-ABL*-positive cells in total *BCR-ABL*-positive cells, a measure of the absolute number of mutant *BCR-ABL*-positive cells is needed. To obtain an estimate of the absolute number of mutant *BCR-ABL*-positive cells, we introduced the size of a mutant clone as $BCR\text{-}ABL^{mut}/GUS=(BCR\text{-}ABL^{total}/GUS)^*$ proportion of mutant transcripts. Assuming a similar transcriptional activity of mutant and unmutated clones the size of a mutant clone gives an absolute quantification of the number of mutant *BCR-ABL*-positive cells.

The median size of mutant clones was 12% (range, 2-60%) before and 1.8% (range, 0-55%; P=0.0003) after cessation of TKI treatment, corresponding to a median relative decline of 86% (range, -34-100%, Table 3). *GUS* quantification was not available for two patients (one with a T315I mutation, the other with a M351T mutation).

With regards to the size of the mutant clones instead of the proportion of mutant transcripts, there was less difference in the median decline after treatment cessation among the different subgroups of mutations (Figure 2B). The median relative decline of the size of mutant *BCR-ABL-positive* clones ranged from 70% to 97% for the different subgroups of mutations (Table 4).

Two patients (patients # 2 and 9) showed a pattern of repeated selection and deselection of the mutant clone after resumption and second cessation of TKI treatment (Figure 3).

Discussion

BCR-ABL mutations represent the major cause of resistance after TKI therapy.[3,4] Here we describe the dynamics of mutant *BCR-ABL* transcripts for five common mutations in 20 cases of TKI cessation. The majority of patients showed a substantial decline of mutant *BCR-ABL* expression within months. This demonstrates that after stopping the selective pressure of the TKI the *BCR-ABL* mutation confers a proliferative disadvantage. The cases of repeated

Table 4. Median follow-up, time to lowest proportion of mutant transcripts, relative decline of the proportion of mutant transcripts, and relative decline of the size of mutant clones for the different subgroups of mutations. The relative decline of the proportion of mutant transcripts reflects the relative decline of the proportion of mutant *BCR-ABL*-positive cells in total *BCR-ABL*-positive cells. The relative decline of the size of mutant clones reflects the relative decline of the absolute number of mutant *BCR-ABL*-positive cells.

BCR-ABL mutation	Y253F	Y253H	E255K	T315I	M351T	Median
Number	1	4	5	8	2	20
Follow-up (months)	26	17	10	6	20	13
Time to lowest proportion (months)	26	6	2	6	13	6
Relative decline of proportion (%)	93	97	93	52	58	88
Relative decline of size (%)	89	97	85	89	76	86

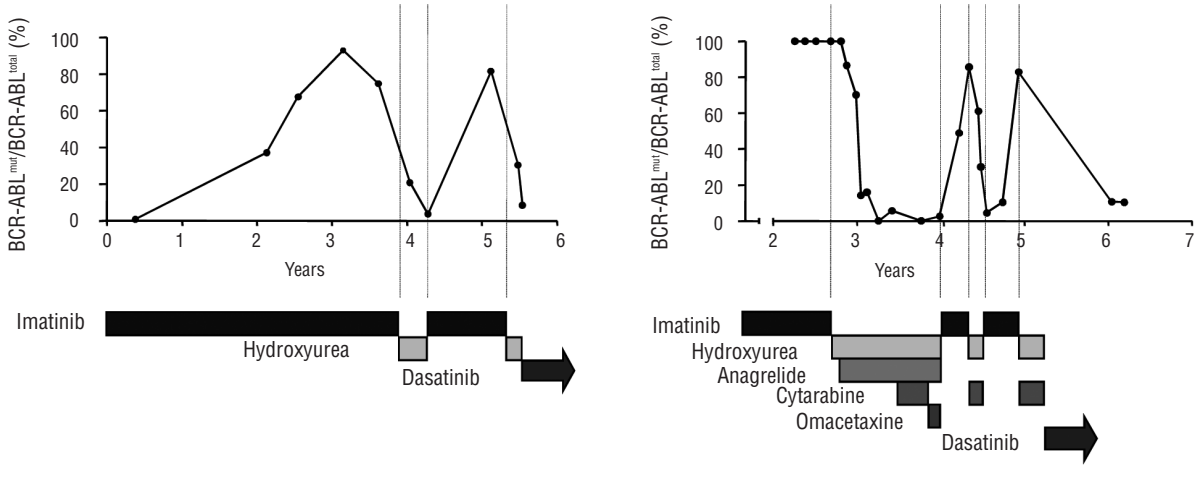

Figure 3. Example of repeated selection and deselection. Patient # 9 (left, mutation E255K) and patient # 2 (right, mutation Y253H) received an alternating regimen with TKI treatment and chemotherapy. Dotted lines indicate onset and stop of TKI treatment. The mutant *BCR-ABL*-positive clones were outgrown by the expanding unmutated *BCR-ABL*-positive clone under chemotherapy. After resumption of TKI treatment clonal selection occurred. Patient # 2 showed deselection under dasatinib at the end of follow up.

selection and deselection suggest that competing clones show a high susceptibility to the onset of TKI pressure as well as to its withdrawal. However, it has been demonstrated that clonal selection does not always occur.[10,21] Indeed, in some patients we observed persistence of mutant clones. Additional factors might influence the impact of the proliferative advantage of either clone. In our series three patients harboring the T315I mutation expressed 100% mutant transcripts until the end of follow-up, whereas in two other patients this mutation disappeared completely. The deselection of a T315I mutant clone after cessation of imatinib had previously been shown in a single patient.[14] A gain-of-function of the T315I mutant BCR-ABL tyrosine kinase, which might explain the persistence of the clone, has been discussed, remaining controversial.[17,18] Compared to the P-loop mutations examined (Y253F/H, E255K) the median decline of the proportion of mutant transcripts was lower with the T315I and M351T mutations (Figure 2A). This difference disappeared with regard to the size of mutant clones (Figure 2B). Some patients showed a decline of the size of the mutant clone due to a declining *BCR-ABL/GUS* ratio while the proportion of mutant transcripts remained persistently high (patients # 11 and 13). Recently, it has been shown that the MAPK pathway is preferentially activated by imatinib in T315I mutated cells as compared to unmutated cells.[27] These *in vitro* data suggest that TKI treatment confers the mutant cell clone with increased oncogenic fitness which is lost after cessation of treatment with the TKI. This might explain the decreasing *BCR-ABL/GUS* ratio after switching to chemotherapy besides the resumption of an effective therapy.

Data on non-specific salvage therapy are important for understanding the activity of second- and third-generation TKI.[22,23] The reduction of mutant clones after TKI withdrawal might be due to non-specific effects or the activity of the new TKI administered.

These results are of particular importance for the activity of drugs addressing the T315I mutation.[28-30] Since we observed the decline of T315I mutant transcripts to a level below the limit of detection of denaturing high performance liquid chromatography (patient n. 17) the ability of the mutant clone to regrow during treatment with another TKI remains open.

We conclude that the deselection of mutant *BCR-ABL*-positive clones within months after discontinuation of TKI treatment is a common and reproducible phenomenon. The decline of the proportion of mutant transcripts seems to be less frequent in patients harboring the T315I mutation than in patients with mutations of the P-loop region. However, there is hardly a difference in the regression of the calculated absolute amount of mutant transcripts. The high susceptibility of competing clones to changes in selective pressure emphasizes the need for early screening for *BCR-ABL* mutations in suboptimally responding patients. Discontinuation of TKI treatment might be an option for patients harboring the T315I mutation. The question of whether treatment discontinuation leads to an eradication of the mutant clone or a persistent mutant clone with the probable consequence of repeated clonal selection after resumption of TKI treatment is object of further investigations using highly sensitive methods.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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