tors such as toxicity and cost need to be considered.

With the current chelation regimen, the balance between iron accumulation and excretion is fine.[1,6,10] In contrast to the iron balance achieved by monotherapy with either DFP or DFO, iron balance achieved with combined therapy was negative in the majority of patients.

In conclusion, combined therapy with DFO and DFP showed an additive and occasionally synergistic effect on UIE, which could reach levels higher than iron accumulation from transfusions, leading to a negative iron balance. Long-term studies are required to validate the efficacy and safety of combined therapy.

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Lack of *Bcr-Abl* point mutations in chronic myeloid leukemia patients in chronic phase before imatinib treatment is not predictive of response

We describe the presence of abl point mutations detected using a highly sensitive technique in 5 out of 9 patients with chronic phase CML resistant to imatinib. These mutations were not detected in samples obtained before initiating therapy with imatinib. Unless more sensitive techniques are developed, the presence or absence of point mutations before starting imatinib therapy will not help in predicting responses to treatment.

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Despite the positive results of treatment with imatinib mesylate (IM) in patients with chronic myelogenous leukemia (CML)[1] a number of patients develop clinical resistance to this drug, resulting in progression of the disease at 18 months in 11% of interferon resistant/intolerant patients.[2] Most patients in blast crisis will eventually suffer disease progression despite continuous treatment with imatinib.[3,4]

Among the different mechanisms of *in vivo* resistance to IM, the most frequently detected in patients with advanced phase (accelerated or blast crisis) CML is point mutations in the kinase domain of *Abl*.[5-7] We studied the presence of *Bcr-Abl* mutations in a homogeneous group of CML patients in chronic phase with primary cytogenetic resistance to IM in order to determine the incidence of point mutations and whether the presence of these substitutions before treatment could predict resistance to IM therapy.

We studied a group of 89 patients with CML enrolled in an extended access trial of IM (chronic phase CML patients resistant to or intolerant of interferon-α). All patients had 100% Philadelphia positive metaphases. Patients with no cytogenetic response after at least 6 months of therapy were defined as having primary resistance to IM and analyzed for the presence of *Abl* mutations. Bone marrow mononuclear cells were obtained before initiating treatment with IM and every 3 months thereafter.

Total RNA was extracted using RNeasy®Mini Kit (Qiagen, Hilden, Germany) from frozen cells. Total RNA (1 μg) was used for cDNA synthesis using SuperScript™ II RNase H-RT (Invitrogen Life Technologies, Paisley, UK) with random hexamers. A BCR-ABL transcript of 1.3 kb was amplified by PCR using 4 μL of cDNA and CM10 (5´-GAAGCTTCTCCCTGACATCCGT-3´) and 3ABL2 (5´-CGGACTTGATGGAGAACTTG-3´) primers under the following conditions: 94°C for 10 min, 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 90 seconds, and a final elongation cycle at 72ºC for 10 min. The Abl kinase domain was amplified in a second PCR using 1 μL of the first PCR product and 5ABLKD (5´-GCGCAACAAGCCCACTGTCTATGG-3´) and 3ABLKD (5´-GCCAGGCTCTCGGGTGCAGTCC-3´) primers with the following conditions: 94°C for 10 min, 30 cycles at 94°C for30 seconds, 70°C for 30 seconds and 72°C for 30 seconds, followed by an elongation cycle at 72°C for 10 min. All PCR reactions were carried out in a total volume of 25 μL, with 2.5 U of native PFU polymerase (Stratagene, Amsterdam, The Netherlands), 0.4 mM dNTPs and 20 pmol of each primer.

The second PCR product (597 bp) was subcloned into pCR® 4-

Table 1. BCR-ABL kinase domain mutations detected in 5 CML patients.

	Nucleotide	*Nucleotide substitution*	*Amino acid substitution*	*Mutant clones*
CML 1	749	G→A	G250E (Gl→Glu)	8/10
CML 2	749	G→A	G250E (Gly→Glu)	10/10
CML 3	730	A→G	M244V (Met→Val)	4/10
	1052	T→C	M351T (Met→Thr)	5/10
CML 4	1075	T→G	F359V (Phe→Val)	10/10
CML 5	1075	T→G	F359V (Phe→Val)	10/10

TOPO® plasmid using TOPO TA Cloning® Kit for Sequencing (Invitrogen Life Technologies, Paisley, UK). Ten colonies with recombinant plasmid containing the 597 bp PCR product were sequenced using T7 and T3 universal forward and reverse primers. Only substitutions present in at least 2 clones were considered as mutations in accordance with Shah *et al*.[6]

Nine of 89 patients showed complete cytogenetic resistance to IM (100% Ph positive metaphases after more than 6 months of treatment) despite having a complete hematologic response. No *BCR-ABL* gene amplification was detected by fluorescent *in situ* hybridization analysis at the time of resistance in any of our patients (*data not shown*) suggesting that this mechanism is rarely the cause of resistance in patients with CML in chronic phase.

Mutations of the *Abl* kinase domain were identified in 5 patients after treatment with IM (Table 1). The detected substitutions correspond to previously described mutations that conferred resistance to IM. The G250E and M244V mutations, located in the ATP phosphate binding loop (P loop), and M351T, located in the C-terminal loop of the kinase, confer resistance due to changes in the conformation of the kinase domain which prevent it from binding IM. The F359V mutation is located at sites that are in direct contact with IM, impairing binding of the drug without affecting binding of ATP.

The current hypothesis is that mutations within the *Abl* gene are induced throughout the course of the disease and that treatment with IM selects for these clones. Our study was initially designed to try to determine whether detection of point mutations in patients with chronic phase CML could predict response to IM. Unlike other studies[6,8] we were not able to detect mutations in samples obtained before treatment with IM. Different techniques for detecting point mutations have different sensitivities. Direct sequencing can only detect mutations when most of the clones harbor the substitution. By subcloning and sequencing multiple independent clones we can detect mutant clones when these represent about 20% of the total *Bcr-Abl* positive cell population.[6] Allele-specific oligonucleotide PCR may detect mutations even when less than 20% of the cells are mutated, however it can only be applied to specific substitutions and requires different primers to be designed for each mutation.[8] The fact that we analyzed a group of patients in chronic phase, who are less likely to harbor mutations than patients in advanced phase, may also explain differences between our results and those of previous studies.[6,8]

In a small group of patients, samples were analyzed in duplicate using 2 different Taq polymerases with or without proofreading activity. Interestingly, the use of a Taq polymerase without proofreading activity detected multiple substitutions present in single clones, some of which have been described as mutations in patients with resistance to IM. None of these substitutions was found when Taq polymerase with proofreading activity was employed suggesting that these were artifacts introduced in the PCR reaction (*data not shown*).

In 4 patients, the 700 bp region 5' to the kinase domain was sequenced using the same approach. No evidence of additional mutations could be found in these patients either before or after treatment with IM. These findings are in agreement with those of previous studies[6] and suggest that there is no global increase in mutation frequency and further support the hypothesis that only mutations associated with an IM-resistant phenotype would be selected.

In conclusion, by subcloning and sequencing multiple clones we detected point mutations in the kinase domain of *Bcr-Abl* in more than 50% of chronic phase CML patients with primary cytogenetic resistance to IM indicating for the first time that this mechanism of resistance to IM is highly prevalent also in chronic phase CML. The fact that no mutations were found in samples obtained before IM treatment suggest that unless more sensitive techniques can be developed, the presence or absence of mutations will not help to predict resistance to IM.

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