Leukemia (2005) 19, 132–134

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Evidence for D276G and L364I Bcr-Abl mutations in Ph+ leukaemic cells obtained from patients resistant to Imatinib

Leukemia (2005) **19,** 132–134. doi:10.1038/sj.leu.2403453
Published online 28 October 2004

TO THE EDITOR

In this report, we present our experience with 20 patients affected by chronic myelogenous leukaemia (CML) in blast crisis (BC)/accelerated phase (AP) or by Ph+ acute lymphoblastic leukaemia (ALL) treated with Imatinib and in whom resistance to the drug (defined as the inability to maintain a haematological response or as the progression to BC for patients who were in AP at the start of the treatment) developed. These patients were enrolled in registrative or not registrative trials of Imatinib as monotherapy. The results obtained are reported here.

After a follow-up of 40 months after relapse, only one patient survives. She was diagnosed with BC-CML and has been treated with Imatinib for 4 months. The patient obtained a complete haematological response (CHR) of 2 months duration, developed resistance, and underwent an allogeneic-related BM transplant.

The median survival time after the development of resistance is of 6 months, and only one patient survived more than 12 months (excluding the patient who was successfully transplanted).

The patients were analysed to investigate whether the resistance to Imatinib was linked to *BCR-ABL* gene amplification or to mutations in the kinase domain of Bcr-Abl, as reported in previous works.[1]

No evidence of *BCR-ABL* gene amplification was detected in the 20 patients (data not shown) by FISH analysis performed with the LSI *BCR-ABL* double fluorescent probes (Vysis Inc., Downers Grove, IL, USA).

In all, 14 patients could be successfully analysed for BCR-ABL mutations (Table 1). The poor quality of the RNA from samples of six patients led to the exclusion of these patients from the mutation analysis. The sequenced part covers the entire ABL catalytic domain. The analysis was considered positive if at least two clones were identified bearing the same mutation. Seven patients (50%; 95% CI = 26.8–73.2%) scored positive for *BCR-ABL* mutations. The presence of mutations did not cause significant differences in survival (not shown) between the two groups of resistant patients (with mutations and without mutations).

Mutation L364I and D276G were identified in patients 2 and 15, respectively.

Patient 2 was diagnosed with AP-CML in April 1994 and started Imatinib therapy (600 mg/day) in December 1999. At the end of January 2000, a CHR was obtained, followed by a major cytogenetic response in June 2000. In November 2000, the patient relapsed, with 70% blasts in BM and died in December 2000.

Patient 15 was diagnosed with Ph+ ALL in April 2001. A CHR and a complete cytogenetic response were obtained by chemotherapy. The patient started Imatinib (800 mg/day) in August 2001. After 5 months of treatment the patient relapsed, with 70% lymphoid blasts in BM and PB. Death occurred 2 months after the diagnosis of relapse.

Mutation L364I was not previously described in clinical samples; mutations affecting residue 276 were previously theorised by Warmuth *et al*,[2] identified as part of mutational screenings by Azam *et al*[3] in an *in vitro* analysis based on a mutagenesis screening and in a recently published clinical screening.[4]

Mutation D276G was also identified by us in one patient affected by CML in chronic phase and who developed resistance to Imatinib (data not shown).

Mutations D276G and L364I were further investigated.

The clonal analysis of *BCR-ABL* in patient 15 led to the identification of a point mutation at position 827 (a→g) in 7/9 evaluable clones, producing a missense D276G mutation. Since 7/9 clones showed the presence of D276G, we conclude that the majority of blasts present at relapse were carrying this mutation. Mutation D276G was confirmed in 7/14 clones at genomic level.

Transfectants for mutation D276G in Ba/F3 and 32D murine cell lines were tested for Imatinib sensitivity. The proliferation assay in 32D transfectants revealed an IC_{50} for Imatinib of 0.89 μM (Figure 1). Similar results were obtained using Ba/F3 transfectants (not shown). This value is similar to that found by Warmuth and co-workers, even if in that case a double mutant (D276S/E279S) was analysed.[2] The difference between Imatinib sensitivity in mutant D276G and in Bcr-Abl ($IC_{50} = 0.31$ μM) is highly significant ($P < 0.0001$).

While the analysis carried out by Warmuth *et al* was performed on double mutant D276S/E279S, our data indicate that mutation D276G alone is sufficient to reduce *in vitro* sensitivity to Imatinib and to induce resistance to the drug.

Warmuth *et al*[2] recently proposed a model in which H bonds between residues D276–E279 and R386 play an important role in the stabilisation of the inactive conformation of Abl, the one that preferentially binds Imatinib. The authors experimentally tested this theoretical model by generating the Abl double mutant D276S/E279S. This mutant resulted to be less sensitive to Imatinib than Abl wt.

More recently, a second mutation D276V has been reported from a mutagenesis study.[3] Although that the X-ray structure gives only a restricted view on dynamical process intrinsic to tyrosine kinase, the structure of Abl complexed with Imatinib (1IEP) was compared with the previously described one (1FPU) and with the model proposed by Warmuth *et al*. 1IEP contains, at difference with 1FPU, the orientation of amino-acid side chains. The analysis of 1IEP shows (Figure 2) that D276 cannot interact with R386, since it points in the opposite direction. In addition, in the tyrosine kinase cKit the sensitivity to Imatinib is

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Received 24 November 2003; accepted 4 June 2004; Published online 28 October 2004

Table 1 Summary of Bcr-Abl kinase domain mutations detected in patients resistant to Imatinib

Patient no.	*Status at study entry*	*Number of clones containing mutations*	*Bcr-Abl kinase domain mutations*
2	CML/AP	3/10	Leu364Ile
3	CML/BC lymphoid	2/10	Glu255Lys
4	CML/BC myeloid	5/10	Phe359Val
5	CML/BC	6/10	Glu255Lys
6	CML/BC myeloid	6/10	Tyr253Phe
7	CML/AP	6/10	Tyr253His
15	ALL	7/10	Asp276Gly

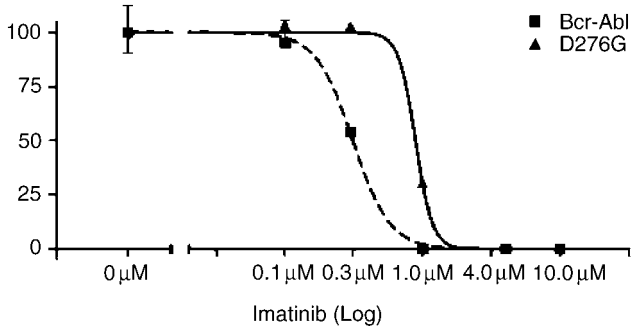

Figure 1 *In vitro* analysis of Imatinib sensitivity in 32D transfectants for mutant D276G compared with Bcr-Abl WT.

conserved, despite the presence of serine at position 276 and arginine at position 386. While these data seem to be in contrast with the mechanistic model proposed by Warmuth *et al*, the hypothesis of a loss of H-bond cannot be ruled out.

Nevertheless, a change from aspartate to glycin not only would cause the loss of H-bond but also increases the conformational entropy of the protein and thus enhances the flexibility of the β3-αC loop (highlighted as red colour in Figure 2). This change directly influences the movement of helix αC that is a key regulatory element for the catalytic activity of tyrosine kinases. Considering the model suggested by Warmuth, the loss of H-bond would influence the movement of helix αC. Thus, we suggest that the D276G mutation as well as other mutations at position 276 could confer resistance to Imatinib by increasing the flexibility of the β3-αC loop; this would destabilise the inactive conformation of the enzyme, as it was reported for other mutations.[2,3,5]

In order to investigate this point, a kinase assay was performed on Bcr-Abl and mutant D276G proteins, previously immunoprecipitated from Ba/F3 transfectant lysates using a polyclonal anti-Abl antibody. This experiment showed that the catalytic activity of D276G is markedly increased compared with the one of Bcr-Abl (Figure 3), while the expression level is unchanged.

A further support to the increased basal activity of mutation D276G is provided by the higher proliferation rate (measured as thymidine uptake) of 32D cells transfected with D276G ($44\,224 \pm 3493$ (s.d.) c.p.m.) in comparison with Bcr-Abl WT transfectants ($30\,789 \pm 4745$ (s.d.) cpm; $P = 0.0038$) after 36 h of growth. Similar results were obtained using Ba/F3 transfectants.

Mutation L364I, corresponding to 1090 (c→a) at the nucleotide level, was identified in three clones on a total of 10. Genomic sequencing analysis confirmed the presence of the mutation in two clones out of a total of 28.

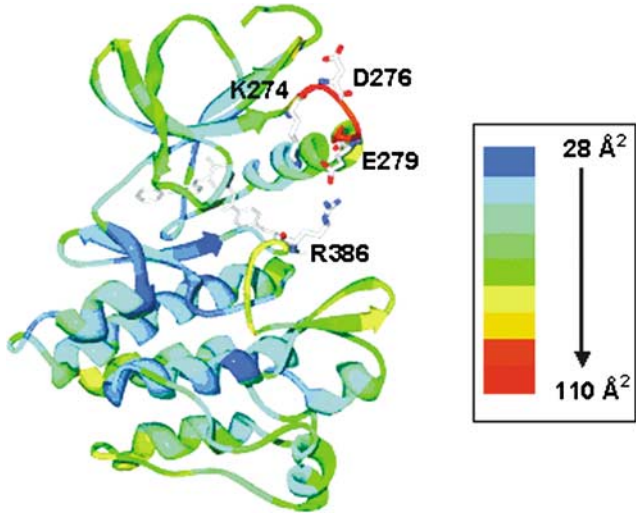

Figure 2 Structural analysis of Abl in complex with Imatinib. Structure colours are coded by structural *B*-factors (blue = low mobility; red = high mobility). The image shows the general mobility of the protein (red = highest mobility), and that D276 is not interacting with R386 but is pointing upwards, toward the solvent.

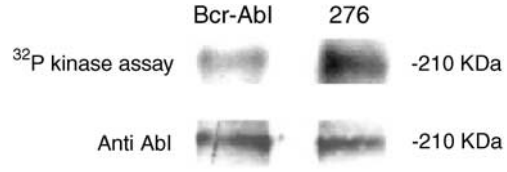

Figure 3 Kinase assay on Bcr-Abl and mutant D276G. A total of 50×10^6 Ba/F3 cells transfected with Bcr-Abl WT or D276G mutant were immunoprecipitated with a polyclonal anti-Abl antibody. The resulting proteins were subjected to a kinase assay using [^{32}P]ATP. The upper lane shows the autoradiographic images. The lower lane represents the bands evidenced by a Western blot assay using a monoclonal anti-Abl antibody.

Transfectants for mutation L364I in Ba/F3 and 32D murine cell lines were tested for Imatinib sensitivity. The proliferation assay on 32D transfectants revealed an IC_{50} of 0.42 μM compared with an IC_{50} of 0.31 μM for Bcr-Abl WT. No statistical evidence for increased cell proliferation, kinase activity or protein levels of Bcr-Abl were evident in the L364I transfectants (not shown).

Amino acid L364 is located in the C-terminal lobe of Abl, in a tight hydrophobic patch (Figure 4). The presence of this hydrophobic patch is required in order to pack helix E, F and I together, thus stabilising the inactive conformation of Abl, while the active conformation of the enzyme can rely on additional interactions.

Although amino acids leucine and isoleucine are chemically similar, structural modelling of the mutant, based on structure 1IEP, reveals that the lateral chain of isoleucine 364 becomes too close to amino acid alanine 426 and a steric clash is generated (Figure 4). In addition, the switch from leucine to isoleucine determines the loss of several hydrophobic interactions among amino acid 364 and F425, F486 and M351 inside the hydrophobic patch. The most likely result of the substitution of leucine with isoleucine at position 364 is therefore the destabilisation of the hydrophobic patch and the destabilisation of the inactive conformation of Abl.

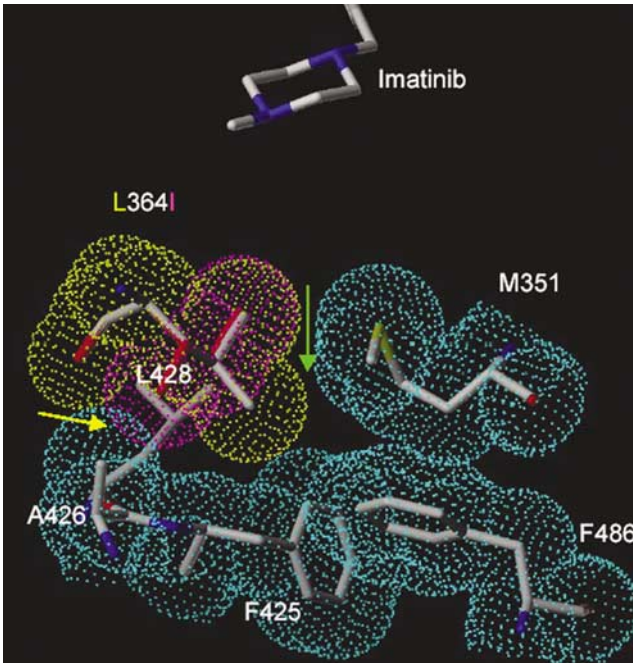

Figure 4 Structural analysis of the hydrophobic patch of Abl WT and mutant L364I. Structural modelling of the mutant L364I (Van der Waals surface displayed as purple dots), based on structure 1IEP, compared with the one of Abl WT (Van der Waals surface showed as yellow dots) reveals that the lateral chain of isoleucine 364 becomes too close to amino acid A426 (yellow arrow) to maintain the usual hydrophobic patch structure in the C-terminal lobe. In addition, the switch from leucine to isoleucine determines the loss of hydrophobic interactions among amino acids 364, F425, F486 and M351 inside the patch (green arrow). The Van der Waals surface of A426, F425, F486 and M351 surrounding position 364 is displayed as cyan dots.

In the present manuscript, we present data obtained in one of the first cohorts of patients treated with Imatinib. The development of resistance represented a poor prognostic sign in this group of advanced, usually heavily pretreated patients, with short survival following the onset of resistance, independent of the length of Imatinib administration or of the underlying disease. Patients carrying mutations in Bcr-Abl have a similarly poor prognosis, when compared to patients with different, yet unknown, mechanisms of resistance to Imatinib;[6] however, the small number of patients precludes a firmer conclusion on this point.

Both mutants were able to induce IL3 independence in the BA/F3 and 32D cell lines; the sensitivity for Imatinib was significantly decreased for mutation D276G, while it was only modestly increased in L364I mutants. It is likely, however, that both these mutations play a causal role in causing resistance. First, they are frequently found in several clones analysed and have been confirmed at genomic level; thus, they cannot represent PCR artefacts. Second, the fact that the level of imatinib sensitivity observed is often barely inferior to that of WT, Bcr-Abl could indicate that the critical parameter for cells in order to resist Imatinib *in vivo* is their capacity to grow at the active concentrations achieved inside cells. It is known that such levels are poor indicators of the amount of drug present inside cells, mostly because of a high affinity binding of Imatinib (and of its major metabolite) to alpha I acid glycoprotein.[7] Therefore, leukaemic cells are exposed to marginally active Imatinib concentrations, and even a modest shift in IC_{50} can lead to resistance. An additional point is represented by the fact that the biochemical/biological results are usually obtained using transfectants in Ba/F3 or 32D cells. These cells could be unable to recapitulate all the effects exerted by the mutant inside the leukaemic cells, such as, for example, the ability to stabilise the protein, thereby increasing protein levels. Notably, mutation D276G was also found by us in a second patient (affected by chronic phase CML, thus not involved in this study). This clearly strengthens the importance of this mutation as a primary factor in the development of resistance to Imatinib.

Mutations at positions 276 and 364 probably are not frequent, since they were found in only two out of 14 patients analysed. Both mutations were associated with an aggressive disease, in spite of the low resistance index. It is however clear from previously studied mutants that resistance index is not proportional to clinical aggressiveness, as demonstrated, for example, by the T315I mutation.[8]

Finally, it has to be recalled that resistant cells maintained sensitivity to Imatinib concentrations that are substantially lower than those observed in the plasma of patients.

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