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Identification of *FOXP1* and *SNX2* as novel *ABL1* fusion partners in acute lymphoblastic leukaemia

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Summary

We have identified two novel *ABL1* fusion genes in two patients with B-cell acute lymphoblastic leukaemia associated with a t(3;9)(p12;q34) and a t(5;9)(q23;q34), respectively. Molecular analysis revealed a *FOXP1-ABL1* fusion for the t(3;9) and a *SNX2-ABL1* fusion for the t(5;9). The fusions were confirmed by specific amplification of the genomic breakpoints using reverse transcription polymerase chain reaction (RT-PCR). The identification of ALL with rare ABL1 fusion partners is important because the leukaemia may respond to tyrosine kinase inhibitors in the same way as ALL patients with a classical *BCR-ABL1* fusion gene.

Introduction

Constitutively activated mutants of the non-receptor tyrosine kinase ABL1 play a central role in the pathogenesis of clinically and morphologically distinct chronic and acute leukaemias (Chase & Cross, 2006). By far the most frequent and best-studied ABL1 fusion gene is *BCR-ABL,* which results from an acquired reciprocal t(9;22)(q34;q11) (Shtivelman *et al*, 1985; Ben-Neriah *et al*, 1986). The t(9;22)(q34;q11) is found in virtually all patients with chronic myeloid leukaemia (CML), in approximately 20% of adults with B-cell acute lymphoblastic leukaemia (ALL), and in rare cases of acute myeloid leukaemia (Fröhling & Döhner 2008). The second most frequent and biologically distinct *ABL1* fusion gene is *NUP214-ABL1* in T-ALL (Graux *et al,* 2004). Remarkably, this fusion is generated by circularisation of the 500 kb genomic region from *ABL1* to *NUP214* and subsequent extrachromosomal (episomal) amplification. Other *ABL1* fusion genes have been described but are uncommon. The *ETV6-ABL1* fusion gene is the product of a t(9;12)(q34;p13) and is found in occasional patients with acute leukaemias or atypical CML (Papadopoulos *et al*, 1995; Golub *et al*, 1996; Andreasson *et al*, 1997). *EML1-ABL1* was found in a single female patient with T-ALL and a cryptic t(9;14)(q34;q32) (De Keersmaecker *et al,* 2005). Recently, *RCSD1*, *ZMIZ1* and *SFPQ* were identified as novel *ABL1* fusion partners in single cases of B-ALL (De Braekeleer *et al*, 2007; Soler *et al*, 2008; Hidalgo-Curtis *et al*, 2008).

Imatinib is a specific inhibitor of several tyrosine kinases including ABL1 and induces long-term complete haematologic and cytogenetic remissions in most patients with chronic phase CML (Druker *et al*, 2001; Deininger *et al*, 2009). However, a substantial proportion of patients with advanced phase CML or *BCR-ABL1* positive ALL are initially refractory to

imatinib treatment or lose imatinib sensitivity over time and relapse (Apperley, 2007; Pui *et al*, 2008). More potent second-generation ABL1 kinase inhibitors (e.g. dasatinib, nilotinib, bosutinib) have been developed for the treatment of imatinib-resistant patients and emerging clinical data indicate that these drugs have promising efficacy (Weisberg *et al*, 2007, Gruber *et al*, 2009). Both, first- and second-generation ABL1 kinase inhibitors are expected to achieve similar remission rates in patients with *ABL1* fusion genes other than *BCR-ABL1*. However, because the majority of these cases have only been reported in individual patients, insufficient data on the *in-vivo* efficacy of tyrosine kinase inhibitors are currently available. *NUP214-ABL* and *EML1-ABL* have been shown to be imatinib sensitive *in-vitro* and two single case reports documented dasatinib sensitivity for *NUP214-ABL* and *RCSD1-ABL in-vivo* (Deenik *et al*, 2009; Mustjoki *et al*, 2009).

In this study, we have investigated two B-ALL patients with acquired chromosomal rearrangements each involving the chromosomal band 9q34 and identified two novel *ABL1* fusion genes potentially sensitive to imatinib treatment. Although these fusion genes occur rarely, their identification is essential in order to detect patients in whom targeted treatment with tyrosine kinase inhibitors is likely to be successful. Furthermore, molecular definition of novel fusion partners for known kinases remains important both for rapid molecular diagnosis of new cases and for monitoring an individual patient's response to treatment.

Patients and methods

Patients

Case 1: *FOXP1-ABL1* male/female B-ALL with t(3;9)(p12;q34).

A 16-year old caucasian woman was diagnosed with pre B cell acute lymphoblastic leukaemia in October 2001, with an initial white blood cell count of >50,000/nL. Her past medical history was significant only for recurrent deep venous thrombosis secondary to protein S deficiency. Bone marrow cytogenetics was 46XX,t(3;9)(p12;q34)[20]. FISH revealed loss of one copy of the p16 locus. Based on her age and an initial white blood count she was considered high risk. Treatment was initiated according to the standard arm for high risk patients of the COALL-97 protocol of the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukaemia (COALL). Therapy was complicated by recurrent DVT, catheter-associated septicaemia and steroid-induced diabetes mellitus. She achieved a complete response and in February 2002 was consolidated with a haplo-identical transplant from her father. Complications included grade III GvHD of the skin and fungal pneumonia, both of which resolved on therapy. On last follow-up in spring of 2010 she continued in CR, with excellent functionality (attending medical school).

Case 2: *SNX2-ABL1* male B-ALL with t(5;9)(q23;q34).

The patient was diagnosed in April 2004 aged 29 when he presented with a WBC of 161 $x10^9$/l. The cell showed a classical B-cell immunophenotype. Bone marrow cytogenetics showed a t(5;9)(q23;q34) and a 20q-. FISH showed evidence of the ABL gene on both derivative chromosomes. BCR was apparently not involved. He responded well to initial

chemotherapy but relapsed early. He then received hyper-C-VAD and again responded. He relapsed again at the end of 2004 and was started on imatinib, it was transient and despite other supportive measures including hydroxyurea he died in Spring 2005.

Fluorescence In Situ Hybridization (FISH)

To our knowledge, no other cases have been reported with a t(3;9)(p14;q34) or t(5;9)(q23;q34). For the t(5;9) FISH was performed using the BCR-ABL probe from Abbott.

5' Rapid Amplification of cDNA Ends (5' RACE) PCR

Partner genes were identified using the Gene-Racer™ Kit (Invitrogen, Paisley, UK). Briefly, ~ 5 µg of total RNA extracted using the Qiagen RNeasy kit (Qiagen, Boundary Court, UK) was dephosphorylated, decapped, and ligated to the GeneRacer™ RNA oligo according to the manufacturer's instructions. The ligated RNA was reverse transcribed using Superscript II™ reverse transcriptase (Invitrogen, Paisley, UK) and random primers (100 ng). Single step 5' RACE PCR was performed using the 5' GeneRacer™ primer from the kit in combination with a reverse primer from ABL1 exon 4 (1R: 5'-cca ccg ttg aat gat gat gaa cc-3'). The PCR cycles were designed to amplify fragments up to 3 kb, with an annealing temperature of 66°C using the High Fidelity PCR Master kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Products were cloned with the TOPO TA Cloning Kit for Sequencing (Invitrogen, Paisley, UK) and sequenced.

RT-PCR Methods

Case 1: The presence of FOXP1-ABL1 mRNA was confirmed on random hexamer reverse transcribed cDNA using primers to FOXP1 exon 18 (5'-gca gta tgg aca gtg gat gaa gta-3') with the reverse ABL1 exon 4 (see above). These primers were also used to amplify the genomic breakpoint.

Case 2: The presence of SNX2-ABL1 mRNA was confirmed on random hexamer reverse transcribed cDNA using primers to SNX2 1F new (5'-aag agt atg tct gct ccc gtg atc tt-3') with the reverse ABL1 exon 4. These primers were also used to amplify the genomic breakpoint.

Results

Characterization of the t(3;9)(p14;q34)

FISH using the probes that immediately flank the ABL1 gene hybridized to the normal copy of chromosome 9, also the der(9) and the der(3), indicating that the translocation targeted ABL1; however, no BCR-ABL1 fusion was seen by FISH or RT-PCR. Most ABL1 fusions reported to date result in the partner gene fusing to ABL1 exons 2 or 3. To identify the t(3;9) partner, we, therefore, performed 5'-RACE PCR from both these exons. These initial attempts failed despite the fact that normal 5' ABL1 sequence was readily obtained (data not shown). 5' RACE primers were subsequently designed in ABL1 exon 4 (ENST00000318560). Sequencing of the products revealed several clones in which FOXP1 (ENST00000318789) exon 19 was fused in frame to ABL1 exon 4.

Confirmation of the FOXP1-ABL1 fusion

The presence of the FOXP1-ABL1 fusion was confirmed initially by RT-PCR. The reciprocal ABL1-FOXP1 product was detectable by single step PCR. Cloning and sequencing revealed

that the reciprocal product fuses ABL1 exon 3 to an alternative RNA isoform (NM_032682) of FOXP1. As shown in Figure 1a and 1b, the FOXP1-ABL1 fusion was specifically amplified from patient cells by single step PCR, but was not detectable in normal controls. To further confirm the presence of the fusion the genomic breakpoint was amplified by long PCR, cloned, and sequenced.

Characterization of the t(5;9)(q23;q34)

FISH using the Abbott BCR-ABL1 probes that immediately flank the ABL1 gene hybridized to the normal copy of chromosome 9, also the der(9) and the der(5), indicating that the translocation targeted ABL1; however, no BCR-ABL1 fusion was seen by FISH or RT-PCR. 5' RACE PCR form ABL1 exons 2 and 3 failed, after the previous cloning of the t(3;9) translocation, 5'-RACE PCR primers were designed in ABL1 exon 4 (ENST00000318560). Sequencing of the products revealed several clones in which SNX2 (ENST00000379516) exon 3 was fused in frame to ABL1 exon 4.

Confirmation of the SNX2-ABL1 fusion

The presence of the SNX2-ABL1 fusion was confirmed initially by RT-PCR. The reciprocal *ABL1-SNX2* product was not detectable by single step PCR. As shown in Figure 2a and 2b, the *SNX2-ABL1* fusion was specifically amplified from patient cells by single step PCR, but was not detectable in normal controls. To further confirm the presence of the fusion the genomic breakpoint was amplified by long PCR, cloned, and sequenced (data not shown).

Discussion

This study has characterised the genomic breakpoints in two B-ALL cases with chromosome 9q34 rearrangements and identified *FOXP1* at 3p14 and *SNX2* at 5q23 as novel *ABL1* fusion partners. This brings the number of known *ABL1* partner genes to nine. In the chimeric protein, N-terminal sequences of ABL1 are replaced by amino-acids derived from the partner protein that provides dimerisation motifs essential for the transforming activity of ABL1. Fusion genes involving *ABL1* are excellent drug targets as exemplified by the activity of imatinib in *BCR-ABL1* positive diseases, particularly CML. The clinical course of patients with *ABL1* fusion genes other than *BCR-ABL1* may be different and highly dependent on the underlying disease. Response rates and risk of relapse may be similar to CML in patients with chronic phase myeloproliferative neoplasms or similar to *BCR-ABL1* positive ALL in patients with acute leukaemias, respectively. As the fusions described here were referred specifically for investigation of 9q rearrangements and were not part of a series that underwent systemic cytogenetic investigation, it is not possible to accurately determine the frequency of these abnormalities. However, we estimate that they probably account for <1% of ALL cases.

The *ABL1* gene is located on chromosome band 9q34 and encodes a ubiquitously cytoplasmic and nuclear protein tyrosine kinase that has been implicated in processes of cell differentiation, cell division, cell adhesion, and stress response. Simplified, the structure of *ABL1* consists of N-terminal SH3 and SH2 domains (which mediate binding to proline-rich and phosphotyrosine proteine ligands, respectively), a catalytic tyrosine kinase domain that contains phosphotransferase activity and a DNA-binding domain with yet largely unknown effects and a C-terminal actin-binding domain. Most previously described fusion genes involving *ABL1* fuse with *ABL1* exon 2. However, similar to the recently described fusion

partners *SFPQ* and *RCSD1* (Hildalgo-Curtis *et al*, 2008; Mustjoki *et al*, 2009) both partner genes described here fuse to *ABL1* exon 4. These are structurally unusual *ABL1* fusion proteins as the SH3 and SH2 domains are not included. Mouse models with BCR-ABL1 constructs in which the SH2 domain was inactivated have shown that the fusion protein retained the ability to cause leukaemia in mice (Roumiantsev *et al*, 2001).

The *FOXP1* gene is located at 3p14 and encodes a member of the forkhead box (FOX) family of transcription factors with diverse functions in development, metabolism, organogenesis and cancer (Hannenhalli & Kaestner, 2009). *FOXP1* was first cloned from the mouse B-cell leukaemia cell line BCL1 and later described to be an essential transcriptional regulator of B lymphopoiesis via direct regulation of the B-cell specific Erag enhancer (Li & Tucker, 1993; Hu *et al*, 2006). *FOXP1* has been described as a recurrent translocation partner of the IGH gene in a subset of MALT lymphomas and diffuse large B-cell lymphomas with t(3;14)(p14;q32) (Streubel *et al*, 2005; Wlodarska *et al*, 2005). Furthermore, *FOXP1* was identified as a translocation partner of *PAX5* in a single case of paediatric B-ALL (Mullighan *et al*, 2007). In contrast to previously published studies, we found *FOXP1* as the 5' partner of the fusion gene. Nearly the entire *FOXP1* gene including the forkhead domain was fused in 5' position to *ABL1*.

The *SNX2* gene is located at 5q23 and belongs to the sortin nexins (SNX) family that function within the endocytic network, including endocytosis, endomsomal sorting and endosomal signalling (Cullen, 2008). *SNX2* is expressed at high levels in CD34 positive bone marrow cells and thought to heterodimerise with *SNX1* in a retromer protein complex. The retromer is a vesicle coat complex that helps in protein sorting between the Golgi and endosomes, playing a key role in protecting some receptors from lysosomal destruction (Seaman, 2005). The hallmark of SNX family is the presence of a PX domain - a sequence of approximately 100-

130 amino acids that has been shown to bind various phosphatidylinositol phosphates, thereby potentially targeting these proteins to specific cellular membranes that are enriched in these phospholipids (Worby & Dixon, 2002). As described above, constitutive activation of ABL fusion genes is mediated by an oligomerisation domain in the partner protein. The *SNX2-ABL1* fusion gene is unusual as it lacks a clearly recognisable dimerisation motif. The N-terminal domain of SNX2 - without the PX domain - is fused to ABL1 exon 4.

Although rare the potential sensitivity to imatinib implies that these fusions should be searched for in acute leukaemia. The screen for such uncommon, but clinically significant fusion transcripts can easily be included in multiplex PCR panels used in routine diagnostic work-ups. Targeted therapy has revolutionised the treatment of CML and there is considerable optimism that targeting *ABL1* fusion genes other than *BCR-ABL1* will bring at least short term benefits for those patients. On the other hand, targeted therapy is very expensive and it will be important to develop markers to identify those patients who are most likely to benefit from treatment.

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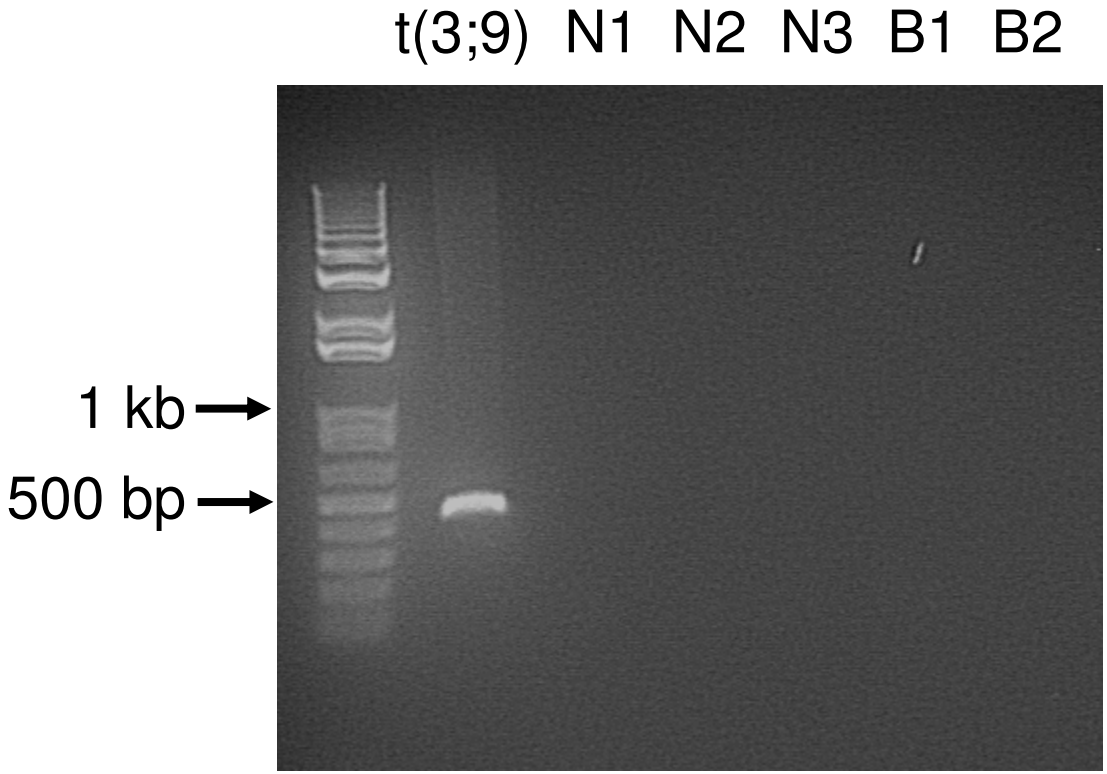
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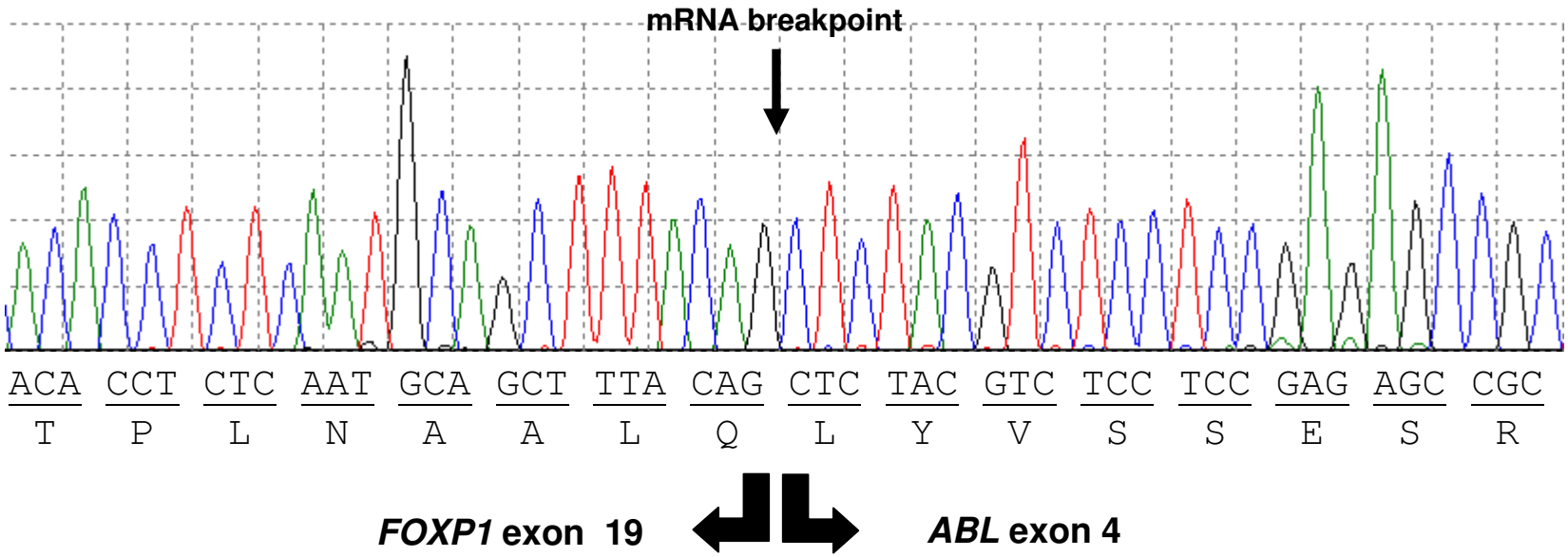
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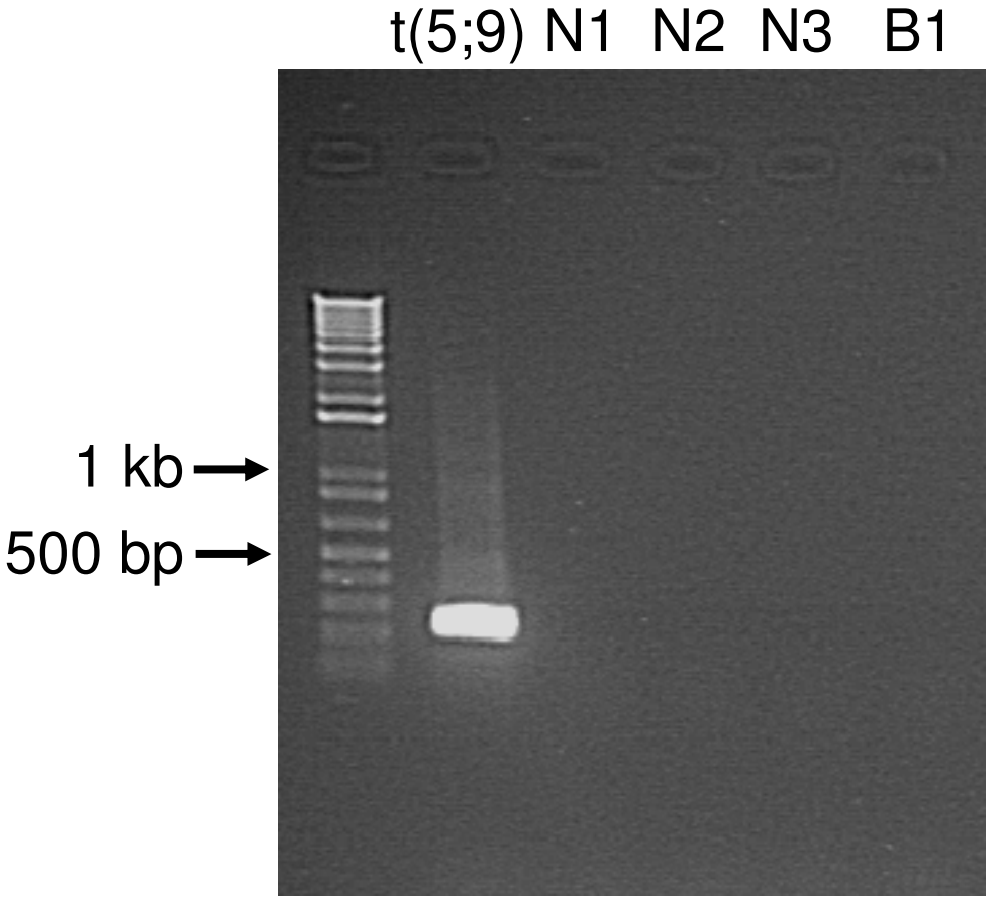
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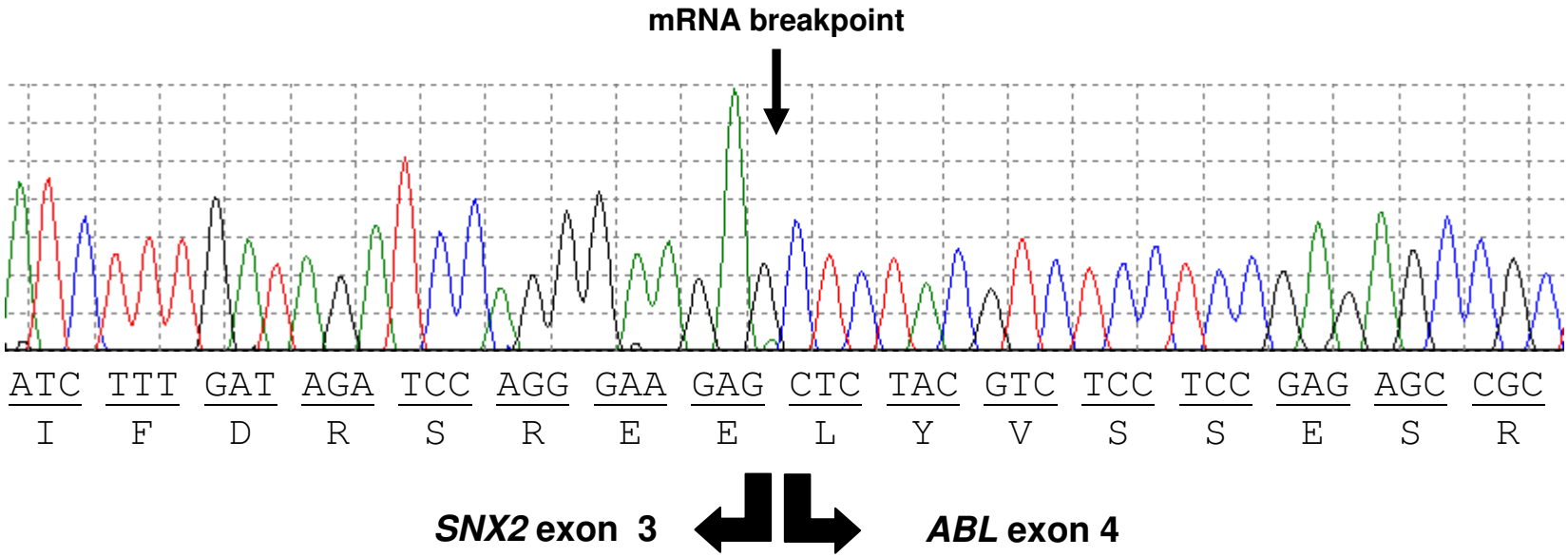
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ABL exon 4