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Low expression of interferon regulatory factor-1 and identification of novel exons skipping in patients with chronic myeloid leukaemia

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Summary. Chronic myeloid leukaemia (CML) is a malignant clonal disorder of the haematopoietic stem cell. Treatment of CML patients with interferon alpha (IFN-α) has induced haematological and cytogenetic remission. Interferons transcriptionally activate target genes through the JAK–STAT and interferon regulated factors (IRFs) family pathways. Interferon regulated factor-1 (IRF-1) is a transcriptional activator of genes critical for cell growth, differentiation and apoptosis. The skipping of exons 2 or 2 and 3 of *IRF-1* in patients with myelodysplastic syndromes and acute myelogenous leukaemia suggests that this factor may have a critical role in leukaemogenesis. The role of *IRF-1* in CML is currently unknown. Therefore, mutational analysis of *IRF-1* was performed and its expression pattern was also studied in CML patients. We studied *IRF-1* in peripheral blood mononuclear cells of 21 patients in chronic phase CML. No point mutations were identified at the cDNA level. Surprisingly, fourfold reduction of full-length *IRF-1* mRNA expression was established in 17/21 patients compared with normal individuals. Low expression of full-length *IRF-1* was observed in conjunction with high levels of aberrantly spliced mRNAs, reported for the first time. In three patients who were also analysed during blastic transformation, further reduction of full-length *IRF-1* mRNA was observed. These findings demonstrate that, in CML patients, *IRF-1* can produce high levels of aberrant spliced mRNAs with subsequent reduction in the levels of full-length *IRF-1* mRNA. This observation is consistent with the notion that exon skipping may constitute another mechanism of tumour suppressor gene inactivation in this disease.

Keywords: IRF-1, chronic myeloid leukaemia, interferon, exon skipping, non-isotopic RNase cleavage assay.

Chronic myeloid leukaemia (CML) constitutes a clonal proliferative disorder of haematopoietic stem cells with expansion of myeloid, erythroid cells and platelets in peripheral blood and myeloid hyperplasia in the bone marrow (Faderl *et al*, 1999). CML usually presents in chronic phase with progression to a fatal blast crisis within 3 to 5 years. An accelerated phase often precedes the blastic transformation (Gordon & Goldman, 1996; Faderl *et al*, 1999). The pathogenesis of CML is a multistep process. The hallmark of the disease is the Philadelphia (Ph) chromosomal t(9;22)(q34;q11) translocation. The molecular consequence of this translocation is the creation of a new fusion protein, BCR/ABL, with active cytoplasmic tyrosine kinase function. The increased tyrosine kinase activity of BCR/ABL protein can phosphorylate several substrates, thereby activating a number of cytoplasmic and nuclear signal transduction pathways that affect the growth and the survival of haematopoietic cells (Sawyers, 1999).

CML is one of the first neoplastic diseases in which therapy with interferon alpha (IFN-α) has been found to be beneficial by suppressing the leukaemic clone (Talpaz *et al*, 1983, 1986; Kantarjian *et al*, 1995). However, the precise mechanisms of this action are still unknown. Interferons elicit their pleiotropic effects through the transcription activation of target genes that possess specific consensus DNA-binding recognition sites within their promoters.

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These genes are regulated through the JAK–STAT pathway and the interferon regulated factors (IRFs) family that posses a broad range of activities (Stark *et al*, 1998; Mamane *et al*, 1999).

The *IRF-1* gene was found to be inducible by both IFN-α/β and IFN-γ and plays a master-regulating role of some interferon-stimulated genes (Stark *et al*, 1998; Taniguchi *et al*, 2001). IRF-1 functions as a transcription activator, whereas the structurally related IRF-2 competes for binding to the same DNA regulatory sequences and functions as a transcription repressor of IRF-1 (Harada *et al*, 1989). *IRF-1* is a tumour suppressor gene and regulates a remarkable spectrum of cellular response. This factor plays a major role in the host response to the viral and bacterial infections, susceptibility to oncogenic transformation, regulation of cell cycle, and is also important for haematopoiesis and immune response (Kimura *et al*, 1994; Taniguchi *et al*, 1997, 2001).

The human *IRF-1* gene has been mapped to the 5q31.1 region (Itoh *et al*, 1991). Deletion of this region is one of the most frequent cytogenetic abnormalities observed in patients suffering from leukaemia and preleukaemic myelodysplastic syndrome (MDS) (Le Beau *et al*, 1986). *IRF-1* was found to be consistently deleted or rearranged in either or both alleles in patients with leukaemia or MDS who exhibited cytogenetic abnormalities in the 5q region (Nimer & Golde, 1987). Thus, loss of *IRF-1* function may be a critical event in the development of human leukaemia. Recent studies have also indicated skipping of exons 2 or 2 and 3 in patients with MDS and acute promyelocytic leukaemia (APL), and consequent loss of the AUG transcription initiator codon (Harada *et al*, 1994; Green *et al*, 1999). These splicing aberrations in the *IRF-1* gene account for the loss of *IRF-1* expression and may constitute another general mechanism of tumour suppressor gene inactivation. Genetic alterations in the *IRF-1* gene have also been reported in other human cancers (Nozawa *et al*, 1998; Park *et al*, 1998; Tada *et al*, 1998). Changes in the relative balance between IRF-1 and IRF-2 lead to dysregulation of cell growth and may play a role in the development of neoplasias. Overexpression of IRF-2 causes the oncogenic transformation of NIH3T3 cells, while concomitant constitutive expression of IRF-1 reverts these cells to a non-transformed phenotype (Harada *et al*, 1993). Low *IRF-1*/*IRF-2* mRNA expression ratio has been reported in patients with acute myelogenous leukaemia (AML) (Preisler *et al*, 2001), while findings of alterations in that ratio in CML patients are controversial (Fischer *et al*, 1996; Hochhaus *et al*, 1997).

In the present study we show that, in CML, the expression of *IRF-1* is reduced and that aberrantly spliced mRNAs are present. These alterations may play an additional role in both the pathogenesis and the progression of the disease.

MATERIALS AND METHODS

Patients' samples. Twenty-one CML patients in chronic phase, positive for BCR/ABL rearrangement were studied. All patients were treated daily with IFNa2b alone or in combination with hydroxyurea and were under partial or complete haematological remission. Eighteen out of 21 patients were studied during the first 14 months after diagnosis (WBC: 9×10^9/l ± 4·144 and median percentage of bone marrow blasts: 3% ± 1·5%), whereas 6 out of the above 18 patients were additionally studied at the time of diagnosis (WBC: $53{\cdot}216 \times 10^9$/l ± 16·171 and median percentage of bone marrow blasts: 5·5% ± 1·6%). In 3/21 patients, *IRF-1* analysis was also performed during blast transformation (bone marrow blasts: 39% ± 6·5%). Ten normal individuals, negative for BCR/ABL rearrangement were also studied.

cDNA synthesis. Total RNA for polymerase chain reaction (PCR) was extracted from patients' and normal individuals peripheral blood mononuclear cells (PBMC) by TRIzol (Gibco – BRL/Life Technologies). After DNase treatment (RQ1 RNase free Dnase kit – Promega, WI, USA), cDNA synthesis was performed using 1 μg of treated RNA and 20 units of AMV reverse transcriptase (Promega), according to the manufacturer's instructions.

NIRCA (non-isotopic RNase cleavage assay). NIRCA analysis was used as an initial screening method to detect mutations of *IRF-1* mRNA (Goldrick *et al*, 1996). Briefly, mutations were detected by ribonuclease cleavage of both strands of duplex RNA obtained by *in vitro* transcription of PCR products containing opposable T7 and SP6 phage promoters. NIRCA was based on previously described protocols (Ritis *et al*, 1998; Speletas *et al*, 2001) and the experimental conditions were carried out according to the instructions of MutationScreener Kit (Ambion – Austin, TX, USA). Amplification of the entire *IRF-1* sequence provides a 1239-bp fragment that is too large to be optimally analysed by NIRCA. To overcome this problem, the 1239 bp fragment was amplified by nested PCR using two sets of primers providing smaller fragments of 713 and 690 bp (positions 68–781 and 574–1264, respectively, of the gene). The positions and sequences of primers, as well as the PCR conditions for NIRCA analysis, are described in the first primers group (Table I).

Expression of IRF1 and IRF2 full-length mRNA. Quantitative analysis of *IRF-1* and *IRF-2* mRNA expression was performed using the QuantumRNA classic II 18S Kit (Ambion). The same primers as those above were used to amplify the entire *IRF-1* mRNA (Table I, second primers group). Because the 1239 bp PCR product of *IRF-1* was usually barely visible on the gel, a nested PCR was performed, providing a product of 1006 bp (Fig 1B). At this point, cycling parameters, linear range and the optimal 18S primer/competimer ratio were optimized according to the manufacturer's instructions. The same strategy was also used for *IRF-2* cDNA amplification. Gels were photographed using a Kodak DC40 camera and the band mean intensity of full-length IRFs and 18S were analysed using Kodak DS, 1D Image Analysis Software, v. 3·0·1 (Eastman Kodak Company, Rochester, NY, USA). The positions and sequences of primers, as well as the PCR conditions for *IRF-1* and *IRF-2* amplification and mRNA expression, are also described in Table I (second and third primers group respectively).

Table I. cDNA amplification of *IRF1* and *IRF2*. Primers sequences and PCR conditions.

Amplified cDNA sequence	Name and sequences of primers	Positions* of primers	PCR product	PCR conditions†
IRF1 NIRCA (1st primers group)	IRF-1 U 5′ GGCGGCCTTAAGAACCAG 3′ IRF-1 L 5′ ACTTGGCAGTGGGGTCACA 3′	59–76 1280–1298	1239 bp	Primary PCR: Denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 90 s, annealing at 59°C for 90 s and extension at 72°C for 90 s.
	‡TIRF-1Ua 5′ AAGAACCAGGCAACC 3′ SIRF-1La 5′ CAGTCGGGGAGAGTG 3′	68–83 767–781	713 bp	Nested PCR (5′ end fragment): Denaturation at 94°C for 5 min followed by 28 cycles of denaturation at 94°C for 75 s, annealing at 62°C for 60 s and extension at 72°C for 70 s
	TIRF-1Ub 5′ AGTCCAGCCGAGATGC 3′ SIRF-1Lb 5′ GCCCACAGAAGTCCA 3′	574–589 1250–1264	690 bp	Nested PCR (3′ end fragment): Denaturation at 94°C ;for 5 min followed by 25 cycles of denaturation at 94°Cfor 75 s, annealing at 62°C for 60 s and extension at 72°C for 70 s.
IRF1 mRNA expression (2nd primers group)	IRF-1 U 5′ GGCGGCCTTAAGAACCAG 3′ IRF-1 L 5′ ACTTGGCAGTGGGGTCACA 3′	59–76 1280–1298	1239 bp	Primary PCR: Denaturation at 94°C for 5 min followed by 32 cycles of denaturation at 94°C for 90 s, annealing at 59°C for 90 s and extension at 72°C for 90 s. Four µl of cDNA were used as template.
	67IRF-1 5′ TAAGAACCAGGCAACC 3′ 1055IRF-1 5′ TGTAGACTCAGCCCAATA	67–83 1055–1072	1006 bp	§Nested PCR: Denaturation at 94°C for 3 min followed by 19 cycles (the middle of the linear range) of denaturation at 94°C for 70 s, annealing at 57°C for 70 s and extension at 72°C for 70 s. One µl of primary PCR was used as template.
IRF2 mRNA expression (3rd primers group)	IRF-2 U 5′ CACACTGAGAGGGCACCAT 3′ IRF-2 L 5′ GCCGACTGCTGCTGGAT 3′	82–100 1058–1074	992 bp	Primary PCR: Denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 50 s and extension at 72°C for 50 s.
	NIRF-2 U 5′ CATGCCGGTGGAAAGGA 3′ NIRF-2 L 5′ GCTGGATGCTGGGGTCA 3′	98–115 1048–1064	966 bp	§Nested PCR: Denaturation at 94°C for 3 min followed by 17 cycles (the middle of the linear range) of denaturation at 94°C for 60 s, annealing at 64°C for 40 s and extension at 72°C for 40 s.

*The nucleotide position corresponds to the cDNA sequences of IRF 1 and IRF 2 reported by Maruyama *et al* (1989) and Harada *et al* (1989) respectively.

†All reactions were carried out in a 50 µl volume using 2·5 units platinum Taq DNA polymerase, 50 pmol of each primer, 200 mmol/l of each deoxynucleotide triphosphate, and 25 mmol/l $MgCl_2$.

‡TIRF and SIRF primers have the sequence of T7 and SP6 promoter, respectively, at the 5′ end.

§This amplification was performed as multiplex PCR adding the optimal ratio 18S primer/competimer.

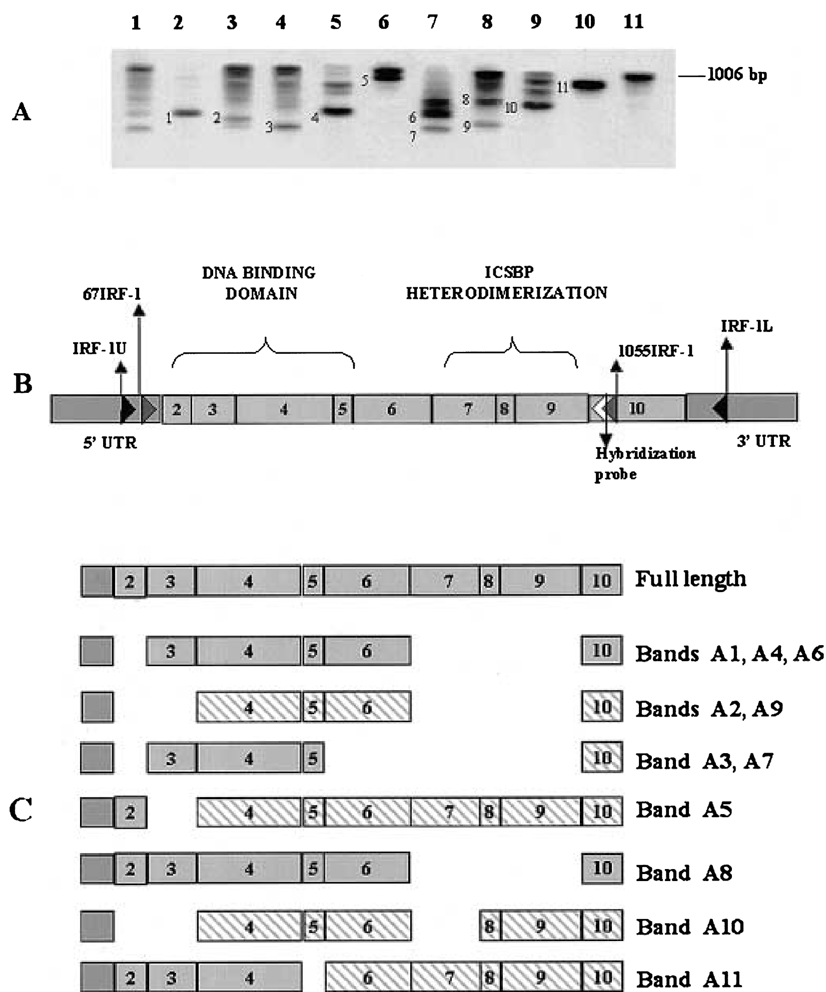

Fig 1. Demonstration of characterized exons skipping of *IRF-1.* (A) Presentation of exons skipping after alkaline transfer and hybridization onto nylon membranes in nine of our patients. Lanes 2–10: CML patients. Lane 1: K562 cell line. Lane 11: normal individual. M: 100 bp ladder (Gibco). Band 1: del2,7,8,9/. Band 2: del2,3,7,8,9/. Band 3: del2,6,7,8,9/. Band 4: del2,7,8,9/. Band 5: del3/. Band 6: 2,7,8,9/. Band 7: del2,6,7,8,9/. Band 8: del7,8,9/. Band 9: del2,3,7,8,9/. Band 10: del2,3,7/. Band 11: del5. (B) Schematic presentation of amplified cDNA *IRF-1.* Arrows indicate primers' positions according to second primers group listed in Table I. (C) Schematic presentation of characterized exons skipping according to bands numeration in (A). Grey boxes indicate unchanged reading frames whereas hatched boxes signify changed reading frames.

Hybridization. Five microlitres of *IRF-1* PCR product obtained using the primers 67IRF1 and 1055IRF1 (Table I) were transferred onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech, UK) by standard alkaline protocol (Sambrook *et al,* 1989). The 5′-ACT-CAGCCCAATATCC-3′ primer (position 1051–1066, Fig 1B), biotinylated at the 5′ end, was used as a hybridization probe. Prehybridization (3 h) and hybridization (12 h) were performed in 6x saline sodium citrate (SSC), 5x Denhart's solution, 200 mg/ml sperm fish DNA and 0·5% sodium dodecyl sulphate (SDS) buffer at 53°C. Subsequently, the membranes were washed in 2x SSC + 0·5% SDS buffer at room temperature for 30 min and then washed twice in 0·5x SSC + 0·1% SDS buffer for 20 min at 58°C. Detection of hybridized sequences was carried out using the PhotoGene detection system (Gibco-BRL, Life Technologies, Paisley, UK) according to instructions of the manufacturer.

Sequencing. Either Exo (–) pfu cyclist DNA sequencing kit (Stratagene, La Jolla, CA, USA) or MWG Biotech AG sequencing service (http://www.mwgdna.com) were used for sequence analysis of PCR products obtained after amplification of *IRF-1* mRNA.

RESULTS

Absence of IRF-1 point mutations

In order to identify putative mutations in the *IRF-1* mRNA by NIRCA, *IRF-1* encoding sequences were amplified by reverse transcription (RT)-PCR. Because no positive control was available, several full-length products were directly sequenced in order to validate our NIRCA results. The sequenced PCR products were negative for mutations and this finding was in accordance with the negative digestion of NIRCA analysis. Subsequently, NIRCA digestion did not reveal any positive findings in the rest of our samples.

Surprisingly, the amplified samples contained primarily the expected size amplification products whereas most of the CML samples contained numerous bands of smaller size. However, of interest was that these smaller products persisted despite our efforts to optimize the PCR conditions and hybridized to each other during the NIRCA procedure. Thus, we hypothesized that these smaller bands were *IRF-1* sequences. In the first PCR–NIRCA fragment, 713 bp (amplified sequence 68–781, Table I), the sequencing of the main smaller bands showed deletion of exon 2 or 2 and 3. Furthermore, the sequencing of some smaller bands

obtained in the amplification of the second fragment, 690 bp (position 574–1264, first primers group, Table I), demonstrated deletion of other exons (except exon 10) in this region. This finding prompted us to examine this issue in the next experiments.

Low expression of IRF-1 and identification of novel aberrantly splicing variants in CML patients

In order to achieve accuracy in the measurement of full-length *IRF-1* mRNA and to characterize possible deletions we developed a nested RT-PCR that amplified a region between exon 1 and part of exon 10 (second primers group of Table I, Fig 1B).

Low expression of *IRF-1* full-length mRNA was observed in 17/21 patients. After comparative normalization of every sample according to 18S RNA expression, the average of full-length mean optical intensity in 10 normal individuals was 14·387 ± 2·508 pixels whereas 17 CML patients had average of mean optical intensity 3·327 ± 1·564 pixels (≈ fourfold reduction of full-length *IRF-1* in the patients). Representative PCR products of 10 CML patients in chronic phase and 10 normal individuals are shown in Fig 2A and B respectively. No obvious changes of *IRF-1* full-length mRNA were observed in six patients that were analysed at the time of diagnosis and 4–7 months after the initiation of IFN-α treatment (data not shown). This showed that the expression of *IRF-1* in the patients was not a direct consequence of the treatment. Four out of 21 patients had *IRF-1* mRNA expression almost equal to normal individuals (data not shown). It is of interest that three of them were treated with IFN-α for more than 4 years. This finding could suggest that prolonged IFN-α treatment of patients restores the expression of full-length *IRF-1* mRNA. However, the short monitoring period of the rest of the patients and the lack of sequential data do not allow us to draw any definite conclusions. Three patients were analysed for *IRF-1* expression in chronic phase and during blastic transformation. As shown in Fig 2C, further reduction of full-length *IRF-1* mRNA was observed during blastic transformation.

Interestingly, the low expression of *IRF-1* in 17/21 patients was associated with the presence of aberrant spliced mRNAs. Figure 1A and C show the aberrantly spliced mRNAs in nine representative patients and the deleted sequences of them are indicated. These novel alterations consisted of skipping of exons 2 or 2 and 3 combined with skipping of exons 6, 7, 8, 9 or 7, 8, 9 or 7 (Fig 1A and C). In two patients, skipping of exon 5 was the main transcript. We also found skipping of exon 3, whereas we could not identify the skipping of exons 2 or 2 and 3 alone. Furthermore, the reading frame of *IRF-1* protein changed when exons 3 and 5 were skipped, leading to a stop codon, as shown in Fig 1C.

Normal individuals expressed the full-length *IRF-1* mRNA. Skipping of exons 2 or 2 and 3 as well as other spliced mRNAs in some healthy individuals were detected after extended PCR amplification (more than 28 cycles nested PCR), whereas in the quantification conditions described here, these deletions were not established (Fig 2B). The same IRF-1 expression pattern was also observed in PBMC and freshly isolated neutrophils, T cells and B cells of three volunteers (unpublished data), indicating that all comparisons have been carried out on CML PBMC versus healthy PBMC, taking into consideration the BCR/ABL status.

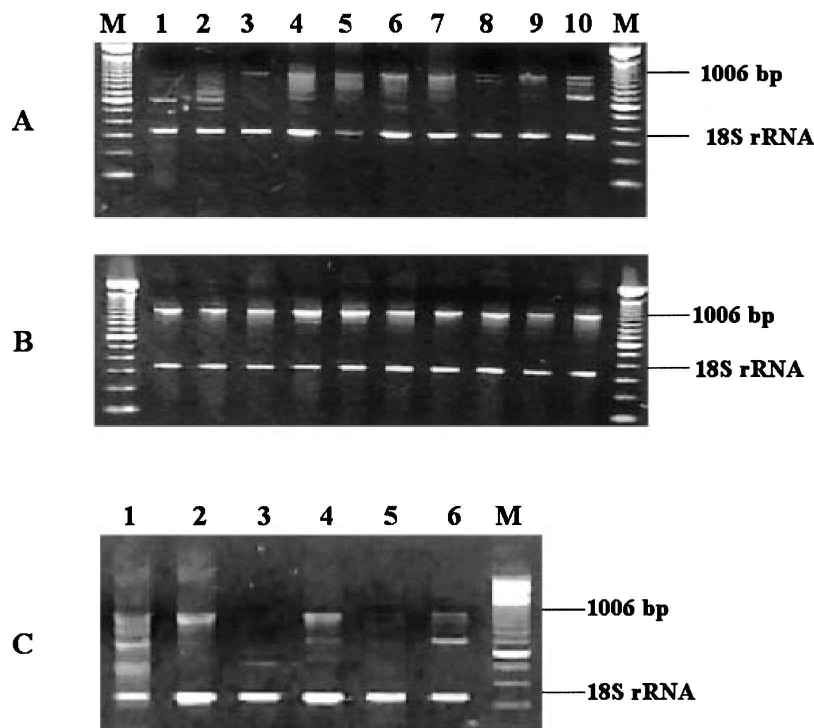

Fig 2. *IRF-1* expression in normal individuals and CML patients. (A) Ten CML patients treated with IFNa2b. (B) Ten normal individuals. (C) Three CML patients analysed both in chronic phase and blastic transformation. Lanes 1, 3 and 5: *IRF-1* expression in blast transformation. Lanes 2, 4 and 6: The same patients as in lanes 1, 3 and 5, respectively, in whom *IRF-1* mRNA expression was analysed in chronic phase. M: 100 bp ladder (Gibco).

IRF-2 mRNA expression in CML patients was similar in comparison with normal individuals and no exons skipping were observed (data not shown).

DISCUSSION

In the present study we examined the expression pattern of *IRF-1* in 21 patients with chronic phase CML. To detect mutations in the *IRF-1* mRNA we used the recently developed NIRCA assay, which we have successfully applied to the rapid mutational analysis of *N-ras* proto-oncogene and *Btk* gene in patients with haematological malignancies (Ritis *et al*, 1998; Speletas *et al*, 2001). Although no point mutations of *IRF-1* mRNA were identified, the amplification of shorter-sized bands than expected that hybridized to each other during the NIRCA procedure suggested the presence of alternatively spliced mRNAs of this gene. Indeed, amplification of full-length *IRF-1* mRNA showed the presence of alternatively spliced mRNAs and a parallel reduction of full-length mRNA.

Recent studies in AML/MDS and APL demonstrated that the human IRF-1 gene can induce exon skipping which generates mRNAs lacking exons 2 or 2 and 3, with the consequent loss of the AUG initiator codon and deletion of all or part of the 5-tryptophan motif, which characterizes the DNA binding region of *IRF-1* protein (Harada *et al*, 1994; Green *et al*, 1999). In this study we established novel exons skipping events and demonstrated that a number of these novel alterations were commonly combined with the previously described skipping of exons 2 or 2 and 3. Thus, in CML, *IRF-1* loses not only the AUG initiator codon and DNA binding domain but also more sequences related to other functions, such as protein–protein interactions. Exons 7, 8 and 9, which are usually deleted in CML, are responsible for the heterodimerization and subsequent activation of ICSBP (interferon consensus sequence binding protein) – another member of the IRF family (Schaper *et al*, 1998). Interestingly, knock-out mice lacking the *ICSBP* gene developed a CML-like disease (Holtschke *et al*, 1996) and very low or absent transcript numbers of *ICSBP* were observed in patients with CML (Schmidt *et al*, 1998).

In parallel with high levels of novel aberrantly spliced mRNAs, low expression of full-length *IRF-1* mRNA was observed in 17/21 CML patients in contrast to normal individuals. A further reduction of full-length *IRF-1* mRNA was also observed in three patients that progressed to blastic transformation. Skipping of exons 2 or 2 and 3 has been proposed as a potential mechanism of *IRF-1* inactivation in patients with MDS and acute promyelocytic leukaemia (Harada *et al*, 1994; Green *et al*, 1999). Additionally, exons skipping as a means of inactivation of gene expression has been also demonstrated to occur in other tumour suppressor genes (WT1, APC, p53), suggesting that subtle alteration of the splicing machinery may selectively affect these ‘error-prone’ genes such as *IRF-1* (Schneider *et al*, 1993; Bala *et al*, 1996; Eicheler *et al*, 2002). As most patients were under therapy with IFNa2b, we cannot convincingly conclude that the observed changes in *IRF-1* mRNA structure are specific to the malignant state and not the result of the treatment. However, there are some evidences that these genetic alterations may be due to cellular differentiation in CML, as no significant changes of *IRF-1* full-length mRNA were observed in chronic phase patients analysed both at diagnosis and few months after IFN-α treatment. Additionally, normal *IRF-1* expression was observed in archival material of bone marrow mononuclear cells from patients with active hairy cell leukaemia, treated for 2–6 months with IFN-α (unpublished data). Importantly, three out of four patients that showed normal levels of *IRF-1* expression received IFN-α for more than 4 years, suggesting that this prolonged treatment ameliorates the expression pattern of *IRF-1*, possibly through suppression of the leukaemic clone. All the above observations raise the possibility that low expression of full-length *IRF-1* mRNA in CML patients, as a result of accelerated exon skipping, may act as an ‘add-on’ to the disease pathology and progression.

Recently, mRNA expression of IFN-α receptor 2c and *ICSBP* – two members implicated in the IFN pathway – has been reported to correlate with the response of CML to IFN-α treatment (Barthe *et al*, 2001; Schmidt *et al*, 2001). In our study, the observed genetic alterations of *IRF-1* could not be directly correlated with responsiveness to interferon treatment as we studied a relatively small number of CML patients, mainly in chronic phase during a relatively short monitoring period. It is obvious that more CML patients need to be studied during the course of their disease, in order to evaluate the possible prognostic role of *IRF-1* in CML.

Although the expression of *IRF-2* mRNA in CML patients was similar to that of normal individuals, in CML patients with very low or an absence of *IRF-1* mRNA expression, however, there was a relative overexpression of *IRF-2*. Considering the fact that overexpression of *IRF-2* in NIH3T3 cells leads to transformation (Harada *et al*, 1993), the imbalanced *IRF-1*/*IRF-2* expression in CML patients could be another event contributing to the pathogenesis of the disease.

In conclusion, *IRF-1* is can produce high levels of aberrantly spliced mRNAs in CML patients, in combination with subsequent reduction of full-length *IRF-1* mRNA. This could constitute another mechanism of tumour suppressor gene inactivation. The prognostic role of *IRF-1* in CML needs to be determined and application of more direct functional characterization of the novel splicing forms, especially those that can potentially encode for truncated *IRF-1* proteins, is required to establish the relevance of our findings for CML pathogenesis.

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