RAPID COMMUNICATION

Lack of Interferon Consensus Sequence Binding Protein (*ICSBP*) Transcripts in Human Myeloid Leukemias

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Interferon consensus sequence binding protein (*ICSBP*) was first identified as a transcription factor of the interferon (IFN) regulatory factor family (IRF) which regulates expression of IFN-dependent genes by binding to DNA at specific sites, IFN-stimulated responsive elements. Analysis of *ICSBP*-deficient mice showed hematologic alterations similar to chronic myelogenous leukemia (CML) in humans and suggested a novel role for *ICSBP* in regulating proliferation and differentiation of hematopoietic progenitor cells. Here we show that *ICSBP*-mRNA expression is impaired in human myeloid leukemias: 27 of 34 CML patients (79%) and 21 of 32 patients with acute myeloid leukemia (AML) (66%) showed very low or absent transcript numbers of *ICSBP*. In contrast, only 2 of 33 normal volunteers (6%) showed low transcription of *ICSBP* ($P < .0001$ both for CML and AML values). The lack of expression was not associated with lack of lymphatic cells, which normally have been shown to express *ICSBP* at the highest level. More detailed analysis showed an absence of *ICSBP*-mRNA also in sorted B cells derived from CML patients. To analyze whether *ICSBP* may be induced in leukemic cells, *ex vivo* experiments using a known inducer of *ICSBP*, IFN-γ, were performed. *Ex vivo* treatment of primary CML cells using IFN-γ resulted in induction of *ICSBP* transcripts. Furthermore, samples of CML patients during IFN-α treatment were analyzed. In 11 of 12 CML patients *ICSBP*-mRNA was inducible upon *in vivo* treatment with IFN-α, but decreased with progression of CML. Stable transfection of K-562 cell line with *ICSBP* led to no difference in *bcr-abl* expression *in vitro*, although two patients showed an inverse correlation between *bcr-abl* and *ICSBP in vivo*. These data suggest that lack of *ICSBP* may have an important role also in human myeloid leukemogenesis.

INTERFERONS (IFNs), divided into type I (IFN-α and -β) and type II (IFN-γ), are cytokines regulating antiviral activity, immune responses, and cell growth in mammals through IFN-regulated genes. The expression of IFN-inducible genes is regulated by IFN regulatory factors (IRFs), which bind to DNA sites containing IFN-stimulated responsive elements (ISRE).[1-3] IFN consensus sequence binding protein (*ICSBP*)[4,5] is one member of the growing family of the IRFs.[6-10] Although some are expressed in many different tissues, *ICSBP* is preferentially expressed in cells of hematopoietic origin. Specifically, highest *ICSBP* expression is detected in mature B cells, while resting T cells and mature macrophages harbor relatively low expression.[11]

It has been shown that mice with deleted *IRF*-1 and *IRF*-2 do not reveal any gross abnormalities.[12] In human leukemias, deletion of *IRF*-1 has been reported,[13] but it has been questioned whether *IRF*-1 was indeed the target gene of the deletion of chromosome 5q, which is common in myeloid disorders.[14] Furthermore, expression of *IRF*-1 and *IRF*-2 has been investigated in chronic myelogenous leukemia (CML) and no abnormality was detected.[15] In contrast to these findings, mice with a null mutation of *ICSBP* exhibit two prominent features: (1) enhanced susceptibility to viral infections; and (2) granulocytic leukemia with enlargement of lymph nodes, liver and spleen, similar to CML in humans.[16] Strikingly, there seemed to be a 'dose-effect' of *ICSBP* in that $ICSBP^{-/-}$ homozygous mice were more prone to blastic transformation of the respective myeloid proliferation compared to $ICSBP^{+/-}$ heterozygous mice.[16] However, in contrast to CML in humans, where the rearrangement of the c-*abl* gene into the *bcr*-gene is observed in more than 90% of the patients,[17,18] no gross genomic alteration of the c-*abl* gene was detected in the leukemic cells of the *ICSBP*-null mice.[16] These data pointed to *ICSBP* as a potential tumor-suppressor gene and a role of *ICSBP* in leukemogenesis.

To address the question whether *ICSBP* may play a role also in human leukemic transformation, we investigated the transcriptional level in human leukemias and normal hematopoietic tissues. Therefore, we used a sensitive semi-quantitative polymerase chain reaction (PCR) assay.

MATERIALS AND METHODS

Patient samples. Patient samples were taken from a single institution (Virchow Klinikum, HU Berlin, Germany). Ten to 20 mL heparinized peripheral blood (20 U/mL) were drawn after informed consent. All acute myeloid leukemias (AMLs) exhibited blast counts above 75%, and acute lymphoblastic leukemias had blast counts above 50%.

Cells and cell lines. Cell lines U-937, Jurkat, and K-562 were obtained from the ATCC (American Type Culture Collection; Rockville, MD) and BV-173 from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Cell lines HL-60, Daudi and Raji were kindly provided by Dr F. Schriever (Virchow Klinikum) and cell lines MOLT-4, EHEB, and DHL4 were gifts from Dr I.G. Schmidt-Wolf (Virchow Klinikum). All cell lines were maintained at 5% CO_2 in RPMI 1640 medium with 1% glutamine (GIBCO-BRL Eggenstein, Germany) supplemented with

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10% fetal calf serum (FCS, GIBCO-BRL), 1% penicillin/streptomycin (Biochrom KG, Berlin, Germany). Mononuclear cells were separated from peripheral blood by centrifugation over a Ficoll (Biochrom KG) gradient and were stimulated with 1,000 U/mL IFN-α (Intron A; Essex Pharma GmbH, München, Germany) or IFN-γ (Boehringer Mannheim, Mannheim, Germany) for 6, 24, and 48 hours, respectively.

Separation of CD19$^+$ B cells. B cells were separated from peripheral blood of normal healthy volunteers and CML-patients using MACS CD19 MultiSort Kit (Miltenyi Biotec GmbH, Sunnyvale, CA) as recommended by the manufacturer. Purity of some B-cell fractions was verified to be approximately 90% using CD19-FITC antibodies (Dako Diagnostika GmbH, Hamburg, Germany) and FACScan analysis (Becton Dickinson, Heidelberg, Germany).

RNA isolation and cDNA synthesis. RNA was extracted from heparinized peripheral blood using the RNAzol-kit (Paesel, Frankfurt, Germany) as recommended by the manufacturer. One microgram of total RNA was heat denaturated at 90°C for 5 minutes in the presence of 100 pmol random hexamers (Pharmacia, Freiburg, Germany) and cooled on ice for 2 minutes. The RNA was reverse transcribed in 20 µL final volume of 1X PCR buffer (Perkin Elmer, Weiterstadt, Germany), 625 nmol/L of each dNTP (Boehringer Mannheim), 10 mmol/L dithiothreitol (GIBCO-BRL), 40 U RNasin (Promega, Madison, WI), and 140 U SuperScript reverse transcriptase (GIBCO-BRL). The reaction mixture was incubated for 10 minutes at room temperature (25°C), 40 minutes at 42°C, and 5 minutes at 95°C.

ICSBP-*mRNA expression analysis by PCR.* PCR was performed using 50 ng single-stranded cDNA in 25 µL 1X PCR buffer, 200 µmol/L dNTP, 500 nmol/L of each primer, and 0.625 U AmpliTaq DNA Polymerase (Perkin Elmer) under following cycling conditions: 94°C for 2 minutes for denaturation; then 94°C for 1 minute, 55°C (for *β-actin*) or 61°C (for *ICSBP*) for 1 minute, 72°C for 1 minute for 21 (for *β-actin*) or 27 cycles (for *ICSBP*), followed by 90°C for 1 minute and 60°C for 10 minutes. The sequences of the primers are as follows: *β-actin* sense primer, 5′-CCTTCCTGGGCATGGA GTCCT-3′; *β-actin* reverse primer, 5′-AATCTCATCTTGTTTTCTGCG-3′, which results in a 407-bp PCR-product; *ICSBP* sense primer, 5′-CAGTGGCTGATC-GAGCAGATTGA-3′; *ICSBP* reverse primer, 5′-ATTCACGCAGC-CAGCAGTTGCCA-3′, which results in a 360-bp PCR product. The products were electrophoresed on a 3% agarose gel. Gels were stained with ethidium bromide and photographed. The gel-photos were scanned and integrated optical densities (IntOD) were calculated using the ONE-Dscan 1.0 software (Scanalytics, Billerica, MA). The ratio 'IntOD *ICSBP*/IntOD β-actin' was then calculated. Analysis of normal control samples suggested a value of 0.200 as cut-off-level.

Other reference genes, porphobilinogendeaminase (*pbgd*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), showed comparable results (data not shown).

Detection of the bcr-abl *translocation and mRNA expression analysis by PCR.* The PCR for detection of the *bcr-abl* translocation was performed as described elsewhere.[19] Quantitative *bcr-abl*-PCR was performed using a competitive differential quantitative PCR-assay which enabled us to determine the relative amplification-equivalence points (EP) of *bcr-abl* and a reference gene, *pbgd*, and to calculate the ratio 'EP bcr-abl/EP pbgd.'[20]

Construction of ICSBP *expression vectors.* An expression-plasmid for *hICSBP* was constructed by cloning a PCR-product of 1,372 bp into pCR3.1 (Invitrogen, NV Leek, The Netherlands). PCR was performed using 50 ng single-stranded cDNA in 25 µL 1X PCR buffer, 200 µmol/L dNTP, 500 nmol/L of each primer, and 0.625 U Expand Long PCR System (Boehringer Mannheim) under following cycling conditions: 94°C for 2 minutes for denaturation then 94°C for 1 minute, 62°C for 1 minute 68°C for 1 minute for 34 cycles, followed by 90°C for 1 minute and 60°C for 10 minutes. The sequences of the primers are as follows: sense 5′-GCGGCGAGACGGCGGCAGGA-3′; reverse 5′-GGCCACT-GTAACAGGGAGATGGA-3′. The primers are based on the recently corrected sequence deposited at GenBank/EMBL Data Bank (accession no. M91196).

Transfection and cloning of stable transfectans. K-562 cells (1.2×10^7) were transfected with 20 µg of control pCR3.1 (without insert) and pCR3.1 containing the coding sequence for *hICSBP* by electroporation with a Gene Pulser II (BioRad, München, Germany). Cells were selected with 0.5 mg/mL geneticin (G-418; GIBCO-BRL) for 2 to 3 weeks and then cloned by limiting dilution. Ten clones propagated from each transfection group were screened by reverse transcriptase (RT)-PCR for *ICSBP*-mRNA expression. Stable transfectans were maintained in culture medium with 0.2 mg/mL geneticin. Three different clones each were used for stimulation experiments with 1,000 U/mL IFN-α (Intron A; Essex Pharma) or IFN-γ (Boehringer Mannheim).

DNA sequencing. *ICSBP*-PCR products and *ICSBP* expression vectors were verified by automated sequencing with the ABI Prism DNA Sequencer 377 (Perkin Elmer) using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) as recommended by the manufacturer.

Statistical analysis. Differences in the *ICSBP* expression of various leukemias were calculated by Fisher's exact test using Statistica 5.0 software (StatSoft, Tulsa, OK).

RESULTS

Determination of ICSBP-*transcript levels in human samples.* We used a sensitive and semi-quantitative RT-PCR-approach to study transcription levels of *ICSBP* in human leukemias, as well as in normal hematopoietic tissues. Data obtained using RT-PCR were confirmed for several samples with the RNase protection assay (data not shown). Due to the restricted availability of RNA amounts from leukemia patients, RT-PCR was used for expression analysis in our further work.

To analyze the number of *ICSBP* transcripts, the relative expression of *ICSBP* was compared to a reference gene, *β-actin.* For both of these genes, PCR protocols were standardized such that the cycle number for each of these genes ensured that PCR-amplification was in its exponential phase. The optimal cycle number for *ICSBP* was determined to be 28 cycles, and 22 cycles for *β-actin.*

In each PCR, three positive controls were run as controls. The data obtained showed reproducible results (coefficient of variation of 16 experiments for three different controls: CV = 12.7%, 9.9%, and 8.1%, respectively).

We also performed control experiments with two other reference genes (*pbgd* and *GAPDH*) which showed comparable results with the data obtained with *β-actin* (data not shown). Therefore, all other experiments were performed using *β-actin* as reference gene.

In addition, we diluted mononuclear cells from a normal healthy volunteer with *ICSBP*-nonexpressing K-562 cells to determine the accuracy of the semi-quantitative RT-PCR. As expected, no linear correlation was found (Fig 1). However, the resulting near exponential curve fitted well for a semi-quantitative assay.

Furthermore, we tested different cell lines with known expression levels of *ICSBP,* which had been previously determined using standard Northern blotting techniques.[5] The results of the RT-PCR approach were in keeping with these results: High *ICSBP*-transcript levels were detected in cell lines Daudi and U-937, whereas K-562, MOLT-4, and HL-60 exhibited no or low *ICSBP* transcripts (data not shown).

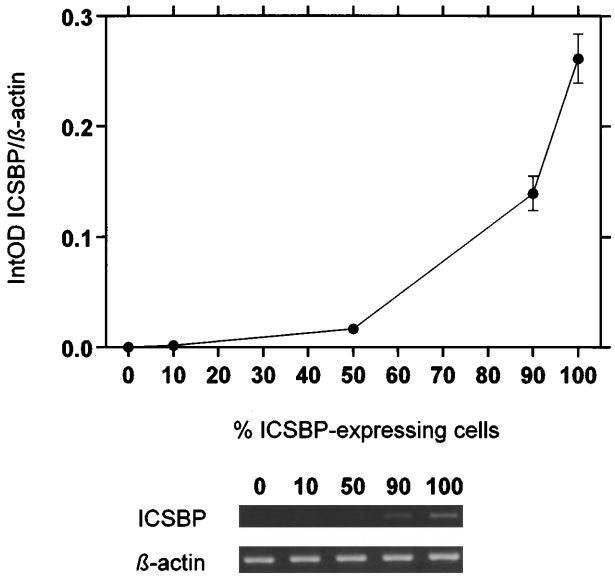

Fig 1. *ICSBP*-transcript numbers in a dilution series of mononuclear cells from a normal healthy volunteer with cells from *ICSBP*-nonexpressing cell line K-562. *ICSBP*-mRNA levels were detected by a semi-quantitative RT-PCR assay. Five different dilutions were done and the percentages of normal cells (*ICSBP*-expressing cells) were shown (0%, 10%, 50%, 90%, and 100%). The mean of three experiments is displayed (±SEM).

Expression patterns of ICSBP *in human leukemias.* To study expression patterns in primary human leukemias, we first determined the level of *ICSBP* transcription in normal, healthy individuals. In 2 of 33 (6%) normal individuals, a relative expression below 0.2 (arbitrary value) was detected (Fig 2 and Table 1). Because $ICSBP^{-/-}$ mice harbor a CML-like disease, we next investigated blood samples from CML patients in chronic phase, all of them *bcr-abl*-positive. Here, very low transcript numbers of the *ICSBP* gene were found in most patients (Fig 2 and Table 1). Twenty-seven of 34 (79%) CML patients showed no or significantly impaired *ICSBP*-transcript levels ($P < .0001$, Fisher's exact test).

For further investigation of myeloid leukemias, samples from patients with AMLs, all *bcr-abl*-negative, were analyzed. Only samples with a blast count above 75% were investigated. In 21 of 32 (66%) of these AML samples, *ICSBP*-transcript levels were low or absent ($P < .0001$). Some AML patients harbored high to very high levels of *ICSBP*-mRNA. Interestingly, these were samples derived from patients with AML-M4 and M5, ie, monocytic differentiating leukemias. In keeping with this, most patients with chronic myelomonocytic leukemia (CMMoL), a disorder classified as myelodysplasia and characterized through monocytic differentiation, exhibited high *ICSBP* levels (Fig 2 and Table 1).

We also analyzed lymphatic neoplasias; none of 11 patients with chronic lymphocytic leukemia (CLL) and 4 of 11 (36%) patients with acute lymphoblastic leukemia (ALL) harbored low values of *ICSBP*-transcript numbers (Fig 2 and Table 1). Out of these 11 ALL patients, all with blast counts above 75%, 3 exhibited high *ICSBP* values. These samples were drawn from patients who suffered from c-ALL, a B-cell–derived disorder. All of our samples from CLL patients, in approximately 95% also a disease of B cells, showed high levels of *ICSBP* transcripts (Fig 2). In keeping with this, all tested B-cell lines (DHL-4, Raji, BV-173, EHEB, Daudi) exhibited high, whereas T-cell lines (Jurkat, MOLT-4) showed low, *ICSBP* values (data not shown).[5]

ICSBP *expression can be induced by IFNs both in vitro and in vivo.* *ICSBP* is inducible *in vitro* with IFN-γ.[4,5] Given that *ICSBP* levels were impaired in CML samples, we first addressed the question whether *ICSBP* may be inducible *in vitro* in $p210^{bcr\text{-}abl}$-positive CML cells. Therefore, leukemic cells of a patient with *bcr-abl*-positive CML were obtained by centrifugation over a Ficoll gradient and incubated *in vitro* for 6, 24, and 48 hours using different IFNs (Fig 3). *ICSBP*-mRNA levels were low at the beginning of the *in vitro* incubation and increased 3- to 10-fold during incubation with IFN-γ, but not with IFN-α. These data suggested that *ICSBP* may be downregu-

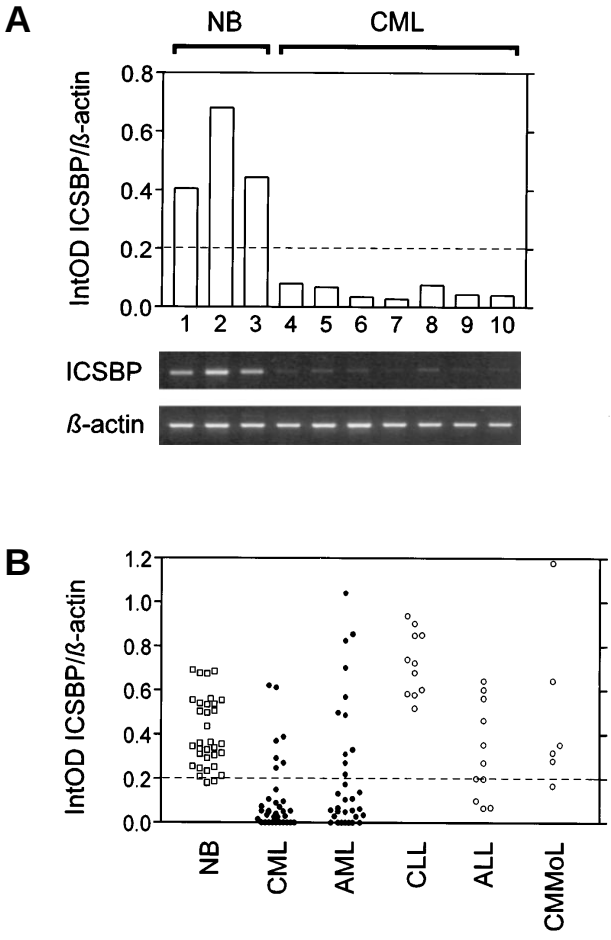

Fig 2. *ICSBP*-transcript numbers in peripheral blood from normal healthy individuals and patients with different kinds of leukemias. (A) Lack of *ICSBP*-transcript numbers in CML-patients was detected by a semi-quantitative RT-PCR assay. Comparison of three healthy normal blood donors (NB; lanes 1 through 3) with four CML patients at diagnosis (CML; lanes 4 through 7) and three hydroxyurea-treated CML patients (CML, lanes 8 through 10). The expression level is displayed as integrated optical density (IntOD). (B) As (A), except that *ICSBP*-transcript numbers of 33 normal healthy blood donors, 34 CML, 32 AML, 11 CLL, 11 ALL, and 6 CMMoL patients were compared.

Table 1. *ICSBP*-Transcript Numbers in Peripheral Blood From Normal Healthy Individuals and Patients With Different Kinds of Leukemias

Diagnosis	Amount of Samples	Mean (CI 95%)*	% Samples With Impaired *ICSBP* Expression†	Fisher's Exact Test
NB	33	0.406 (0.350-0.462)	6	—
CML‡	34	0.113 (0.054-0.171)	79	$P < .0001$
AML§	33	0.225 (0.121-0.330)	66	$P < .0001$
CLL	11	0.725 (0.628-0.822)	0	$P = .5581$
ALL‖	11	0.322 (0.176-0.468)	36	$P = .0269$
CMMoL	6	0.491 (0.100-0.881)	17	$P = .4008$

Abbreviations: NB, normal healthy blood donors; CI, confidence interval.

*Relative *ICSBP* expression is displayed as arbitrary value, calculated as ratio of the integrated optical densities (IntOD) of *ICSBP*- to *β-actin*- transcript numbers.

†The cut-off for normal *ICSBP* expression was fixed at the arbitrary value of 0.200.

‡All CML patients were untreated, treated with hydroxyurea, or exhibit relapses of CML after bone marrow transplantation.

§All AML patients exhibited blast counts above 75%.

‖All ALL patients exhibited blast counts above 50%.

lated in myeloid leukemias, because *in vitro* cultivation with IFN-γ led to a significant increase of *ICSBP* message.

Since IFN-γ induced *ICSBP* in normal and malignant cells in vitro, we asked whether *ICSBP* may also be induced after in vivo treatment with IFN-α, where a survival benefit has been shown in large multicenter trials.[21-23] Therefore, 12 patients were investigated during follow-up. Indeed, *ICSBP*-transcript numbers were significantly increased in 11 of them (91.7%) while patients were receiving IFN-α, but also decreased with progression of CML (Figs 4 and 5, Table 2).

In particular, in one patient, *ICSBP* levels were low at the beginning (0% to 3% blast cells), increased during IFN-α therapy, and decreased during progression to blast crisis (60% blast cells) (Fig 5). These results suggest that *ICSBP* expression is low in chronic and blastic phase of CML; however, it may be induced in cells of chronic phase by IFN-α.

The detected changes in ICSBP-*mRNA levels are not due to variations in the blood differential.* To rule out the possibility that an effect of the peripheral blood differential, especially lymphocyte numbers, accounted for the lack of *ICSBP* transcripts in samples from CML patients at diagnosis and under hydroxyurea, we compared blood counts from different CML patients (Table 3). We found that in three of six selected patients in chronic phase (Table 3, patients 2, 4, and 6), peripheral blood counts and percentage of lymphocytes were near normal. Still, *ICSBP*-transcript levels were significantly impaired or absent, arguing against the possibility that lack of *ICSBP* transcripts in CML was a result of changes in the blood differential. In keeping with this notion was the comparison to CML patients

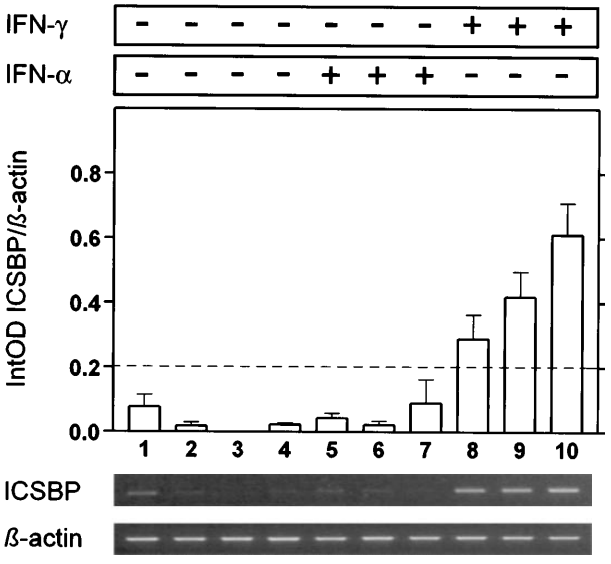

Fig 3. *ICSBP*-transcript numbers in mononuclear cells of a CML patient during *in vitro* stimulation with IFN-α and IFN-γ. An initial control was obtained (lane 1); samples were obtained after 6 hours (lanes 2, 5, and 8), after 24 hours (lanes 3, 6, and 9) and after 48 hours (lanes 4, 7, and 10). No increase of *ICSBP*-transcript numbers was seen without IFN (lanes 2 through 4) or with IFN-α treatment (1,000 U/mL; lanes 5 through 7). Only during IFN-γ treatment (1,000 U/mL) *ICSBP*-transcript numbers increased significantly (lanes 8 through 10). The mean of three experiments is displayed (±SEM).

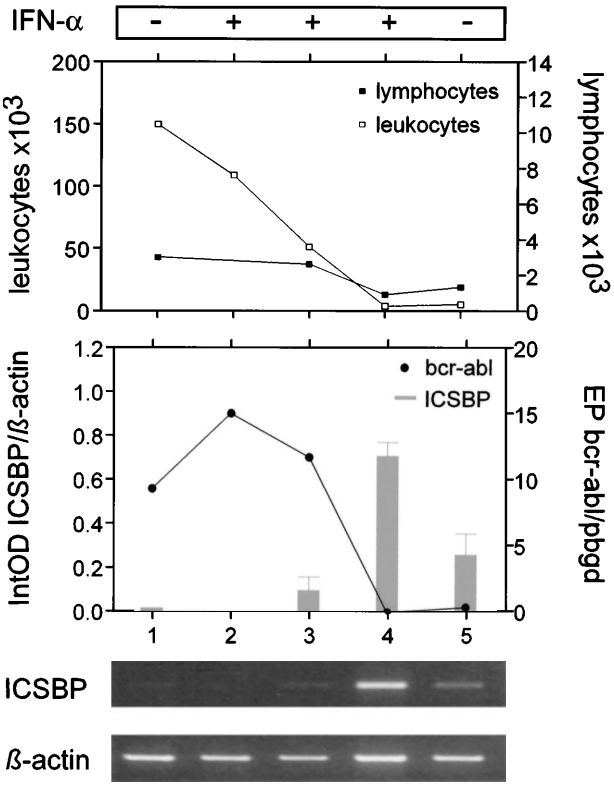

Fig 4. *ICSBP*-transcript numbers, increasing upon *in vivo* treatment with IFN-α, correlated with impairment of *bcr-abl*-transcript (●), leukocyte (□), and lymphocyte (■) numbers. The sample at diagnosis exhibits few *ICSBP* transcripts, even with only 1% blast cells (lane 1). Samples during IFN-α treatment showed an increase of *ICSBP* (lane 2: after 3 weeks, bone marrow; lane 3: 6 weeks; lane 4: 36 weeks), and a decrease of *ICSBP* after IFN-α was withdrawn (lane 5: 68 weeks). The mean of three experiments is displayed. All analyzed metaphases during the patient's course were 100% Philadelphia-chromosome positive. The relative equivalence points (EP) of *bcr-abl* and the reference gene, *pbgd,* were determined by quantitative PCR.

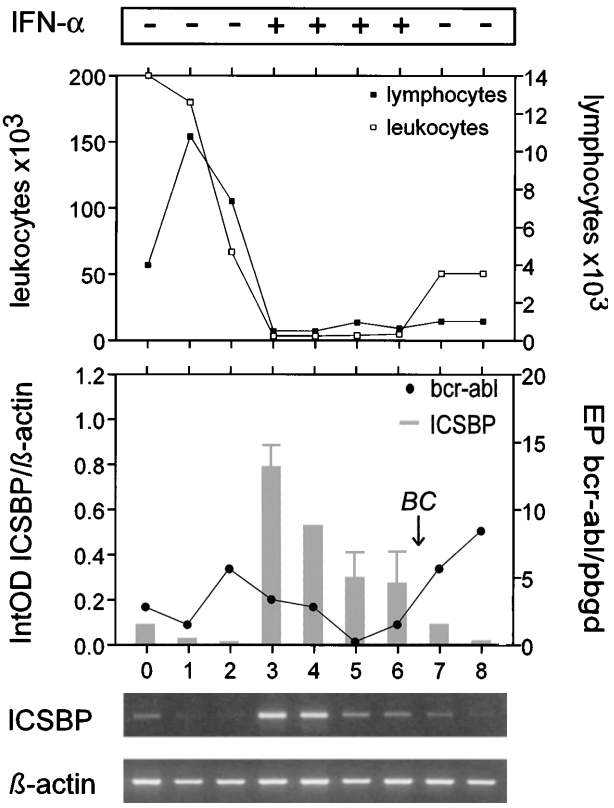

Fig 5. Increase of *ICSBP*-transcript numbers in a CML patient during *in vivo* IFN-α treatment, inversely correlated with *bcr-abl*-transcript (●), leukocyte (□), and lymphocyte (■) numbers. At diagnosis (lane 0), and during treatment with hydroxyurea (lane 1: after 0.5 weeks; lane 2: 1 week) few *ICSBP* transcripts were visible, although in this period the patient exhibited only 0% to 3% blast cells. During early IFN-α treatment *ICSBP*-transcript numbers increased (lane 3: 15 weeks; lane 4: 15 weeks), but decreased with progression to blast crisis (lane 5: 22 weeks; lane 6: 31 weeks; lane 7: 37 weeks, blast crisis with 60% blast cells; lane 8: 37 weeks, blast crisis, bone marrow). The mean of three experiments is displayed (±SEM). The patient exhibited an additional cytogenetic aberration, a monosomie 7, during blast crisis. BC, blast crisis.

under IFN-α: Three of five selected patients (Table 3, patients 7, 9, and 11) with high *ICSBP*-transcript numbers had comparable leukocyte and lymphocyte counts as other patients with low *ICSBP* levels and not receiving IFN-α.

In addition, we compared *ICSBP*-transcript numbers with the amount of leukocytes and lymphocytes in two patients (Figs 4 and 5) during follow-up. The number of lymphocytes decreased in both patients while the *ICSBP* message was upregulated. In one of these patients the relative amount of lymphocytes remained the same while *ICSBP*-transcript numbers decreased during a given period (lanes 3 through 6, Fig 5). The other patient never showed a cytogenetic response during his clinical course, ie, all metaphases analyzed were always 100% Philadelphia-positive (Fig 4), suggesting that an increase of 'normal,' Philadelphia-negative cells was not responsible for the observed increase of *ICSBP* transcripts in this patient.[24]

To investigate alteration of *ICSBP* expression in CML in more detail, we analyzed *ICSBP*-transcript numbers in $CD19^+$ B cells, known to express *ICSBP* at the highest level (Fig 6).

Table 2. *ICSBP*-Transcript Numbers From CML Patients Treated With IFN-α During Follow-up

Patient No.	*ICSBP*-Transcript Levels		
	At Diagnosis	Early IFN-α Treatment	Late IFN-α Treatment*
1	−	+	−
2	−	+	−/+
3	−	+	ND
4	−	−/+	−/+
5	−	+	−
6	−	−	−
7	−	+	ND
8	−	+	−/+
9	−	+	−/+
10	−	+	−
11	−	−/+	ND
12	−	+	−

Abbreviations: ND, not determined; −, no/low *ICSBP* expression; −/+, moderate *ICSBP* expression; +, high *ICSBP* expression.
*Progression of CML.

Seven samples from normal healthy individuals showed high *ICSBP*-mRNA levels whereas two of three samples from CML patients without IFN-α therapy exhibited low ICSBP-transcript numbers (Fig 6, lanes 1 through 10). Another sample, taken from a CML patient previously treated with IFN-α, had a high *ICSBP* level as did one of two samples from CML patients currently under IFN-α (Fig 6, lanes 11 through 13). These data correlated favorably to our results with unsorted peripheral blood cells.

All together these results made it unlikely that the observed lack of *ICSBP* transcripts and the upregulation during IFN-α therapy in CML patients was due to changes in the blood differential, and seemed mainly to be a phenomenon of aberrant *ICSBP* expression in B lymphocytes.

No regulating effect of ICSBP- *on* bcr-abl-*transcript numbers was detected in vitro.* Since levels of *bcr-abl* transcripts have been correlated with progression of the disease, we asked if there may be a inverse association between *bcr-abl* and *ICSBP* expression. In two CML patients we compared *ICSBP*-

Table 3. Comparison of Blood Differentials and *ICSBP*-Transcript Numbers in Selected CML Patients Without or Under IFN-α Treatment

Patient No.	IFN-α Therapy	Leukocytes ($\times 10^3/\mu L$)	Lymphocytes (%)	Blasts (%)	*ICSBP*-mRNA Level*
1	−	137.0	0	15	0.000
2	−	2.7	13	0	0.071
3	−	16.6	11	1	0.150
4	−	4.6	10	0	0.010
5	−	453.0	2	4	0.000
6	−	7.8	17	1	0.170
7	+	3.4	15	0	0.718
8	+	2.3	49	0	0.510
9	+	8.8	8	0	0.710
10	+	5.7	18	0	0.416
11	+	6.8	15	0	0.569

Abbreviations: −, no IFN-α treatment; +, under IFN-α therapy.
*Relative *ICSBP* expression is displayed as arbitrary value, calculated as ratio of the integrated optical densities (IntOD) of *ICSBP*- to *β-actin*-transcript numbers.

expression patterns with the quantitative determination of the *bcr-abl* mRNA.[20] We found an inverse correlation between transcript levels of *ICSBP* and *bcr-abl* (Figs 4 and 5).

To evaluate the possible effect of *ICSBP* on *bcr-abl* expression, we analyzed the relationship between *ICSBP*- and *bcr-abl*-transcript numbers in mononuclear cells from a patient with CML. Untreated cells and cells, which were treated *in vitro* with IFN-α or IFN-γ for 24 hours, showed no significant differences in *bcr-abl* expression, though an increase of *ICSBP* transcripts after incubation with IFN-γ was detected (Fig 7A). To analyze a possible interaction of *ICSBP* and $p210^{bcr\text{-}abl}$ in more detail, we stably transfected the *bcr-abl*-positive cell line K-562 with an *ICSBP*-expression vector. The K-562 cells stably transfected with *ICSBP* were found to express *ICSBP* at high levels (Fig 7B). In these cells no change of *bcr-abl*-transcript numbers was detected compared to *ICSBP*-nonexpressing K-562 cells. Also, after incubation for 24 hours with IFN-α or IFN-γ, K-562 cells exhibited, as expected, nearly the same levels of *bcr-abl*-transcripts (Fig 7B).

Although an inverse correlation was seen *in vivo*, our results suggest no direct regulating effect of *ICSBP* on *bcr-abl* expression, but an effect of *bcr-abl* on *ICSBP* expression could not be ruled out and has to be further investigated.

DISCUSSION

Recently, $ICSBP^{-/-}$ mice have been generated. These mice showed no difference in size, behavior, and reproductive ability as compared with normal littermates. However, in 100% of these mice, a hematologic neoplasia resembling CML in humans was observed.[16] The most prominent features of these mice were hepatosplenomegaly and enlargement of lymph nodes with infiltrations of mature granulocytes, metamyelocytes, and bands. Blood films of these mice showed leukocytosis with enlargement of immature cells and, in keeping with CML in humans, transformation to blastic phase was observed. Interestingly, there seemed to be a 'dose-response' in that homozygous mice were more prone to develop blast crisis as compared with heterozygous mice.[16] These data prompted us to investigate the role *ICSBP* may play in human leukemia.

We used a semi-quantitative RT-PCR approach to determine *ICSBP*-transcript numbers in different kinds of leukemia samples, because no monoclonal antibody was available to study human *ICSBP*-protein expression. The data presented

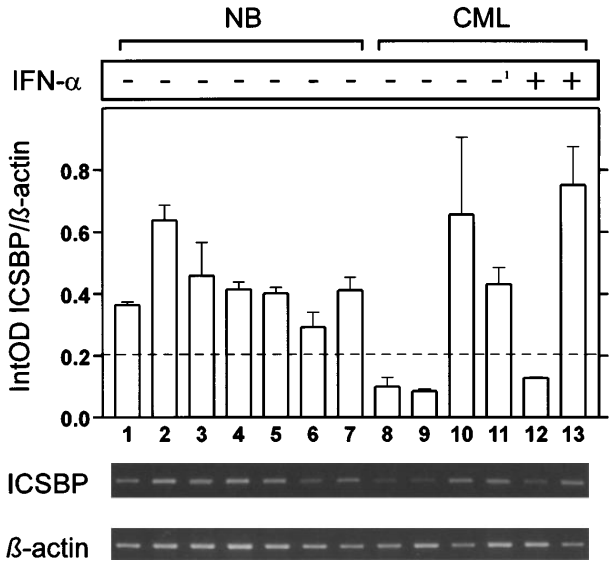

Fig 6. *ICSBP*-transcript numbers in CD19⁺ B cells from normal healthy individuals and CML patients. High transcript levels were detected in cells from normal individuals (lanes 1 through 7), low levels in two of three CML patients without IFN-α (lanes 8 through 10) whereas another patient with high *ICSBP* levels had received IFN-α before (lane 11; IFN-α was withdrawn 6 months before sample was taken). One of two CML patients still under IFN-α exhibited low, the other high *ICSBP* levels (lanes 12 and 13). The mean of three experiments is displayed (±SEM).

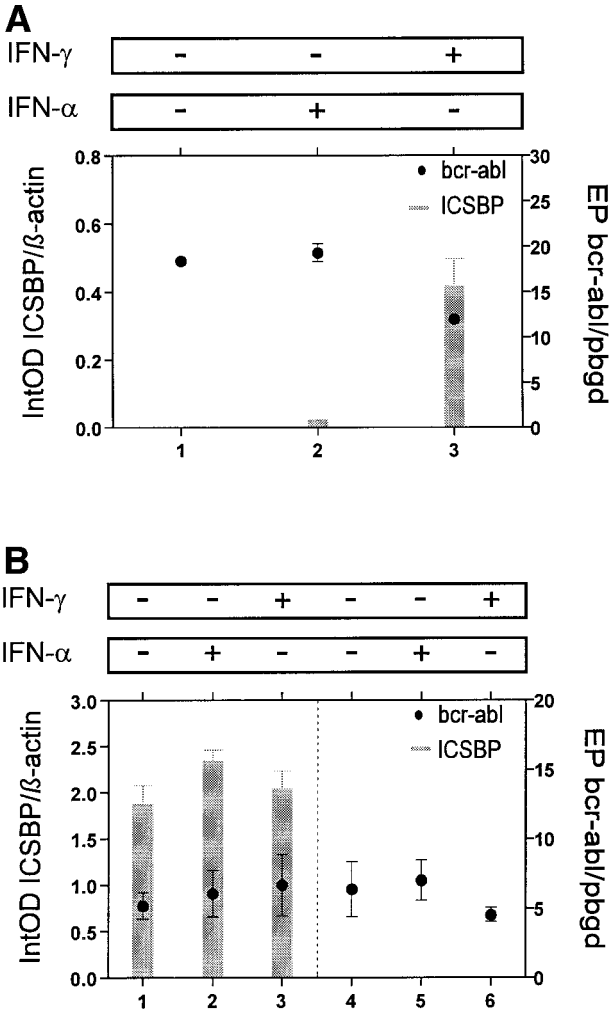

Fig 7. Comparison of *ICSBP*- and *bcr-abl*-transcript levels *in vitro*. (A) Mononuclear cells from a patient with CML were incubated for 24 hours with no cytokine (lane 1), IFN-α (lane 2), or IFN-γ (lane 3). In IFN-γ–treated cells *ICSBP*-mRNA levels increased, but no change was seen with *bcr-abl*-mRNA levels. (B) Stable transfectans of *ICSBP*-expressing (lanes 1 through 3) and nonexpressing K-562 cells (lanes 4 through 6) are compared. Incubation for 24 hours with no cytokine (lanes 1 and 4), IFN-α (lanes 2 and 5), or IFN-γ (lanes 3 and 6) showed no differences in *bcr-abl*-mRNA levels. Experiments were performed with three different transfected clones each and the mean is displayed (±SEM).

here showed that *ICSBP* transcripts are absent or significantly lower expressed in peripheral blood cells in the vast majority of patients with myeloid leukemias. In addition, in some patients with acute lymphoblastic leukemia, mostly T-ALL, *ICSBP* transcripts were also found at lower levels, but not in B-cell–derived disorders like c-ALL or CLL. These data raise the possibility that loss of *ICSBP* expression may play a significant role in human myeloid leukemogenesis. We do not believe that the lack of *ICSBP* transcripts was simply due to expansion of more immature progenitors in the investigated leukemias, because in some of the investigated CML samples a nearly normal blood differential due to treatment with hydroxyurea was observed, yet *ICSBP* levels were still absent, in contrast to samples from CML patients under IFN-α, which had comparable blood counts but high *ICSBP*-transcript numbers. In keeping with this, analysis of $CD19^+$ B cells, normally highly expressing *ICSBP,* showed absence of *ICSBP* transcripts in CML samples.

Furthermore, *ICSBP* was inducible in the leukemic cells, both *in vitro* and *in vivo.* These data suggest that *ICSBP* is not genomically lost but rather downregulated in myeloid leukemias, the mechanism of which remains to be determined. Downregulation leading to gene-'silencing' has been described in human cancer cells for *MTS-1,* a gene coding for an inhibitor of cyclin-dependent kinases (*p^{16}INK-4/CDKN2*).[25,26]

Although patients with CML may respond beneficially to treatment with IFNs, treatment with IFN-γ, in contrast to IFN-α, remains anecdotal.[27-30] In our clinic CML patients were only treated with IFN-α, so we were unable to assess the effect of IFN-γ, a known inducer of *ICSBP*,[4,5] on *ICSBP* expression *in vivo.* In several clinical samples, although many CML patients showed low levels of *ICSBP* transcripts at diagnosis, IFN-α therapy led to an increase in *ICSBP* message. These high levels were also detected in some $CD19^+$ B cells from CML patients under or after IFN-α treatment. The induction of *ICSBP* by IFN-α has not been described *in vitro,* in keeping with data obtained in this study. One explanation for the missing induction of *ICSBP* during IFN-α incubation *in vitro* may be that the increase of *ICSBP* transcripts in CML patients during IFN-α therapy cannot be found within a short period of time, and may take at least 1 to 3 months. Further, the observed upregulation of *ICSBP* during IFN-α treatment *in vivo* may also be an indirect effect mediated by other factors or cells not present in the *in vitro* incubation.

When the patients progressed to accelerated/blastic phase, *ICSBP*-mRNA levels decreased significantly, even during treatment with IFN-α. Thus, this raises the possibility that low or absent levels of *ICSBP* transcripts may be associated with progression of myeloid leukemias, and upregulation may be of some unknown benefit. The importance of some kind of *ICSBP* -'dose-response' for myeloid cell differentiation may also be supported through data from *ICSBP* knock-out mice, in that mice with a complete loss of *ICSBP* progressed to blastic phase in 33% within 50 weeks of observation, whereas heterozygous mice showed acute leukemia in only 9%.[16]

The data of this study make it unlikely that loss of *ICSBP* is necessary for the development of all myeloid leukemias. However, the majority of myeloid leukemias displayed decreased or absent expression of *ICSBP* transcripts. The mechanism by which loss of *ICSBP* may induce leukemias is unclear at present. IFNs play an important role in the negative regulation of human hematopoiesis. In keeping with this, the beneficial role of these cytokines in human myeloproliferative diseases has been shown in recent years. There may be direct repressing effects of *ICSBP* on downstream, positive effector genes. Data obtained in two patients of this study showed that upregulation of *ICSBP*-mRNA was inversely correlated with downregulation of *bcr-abl* transcripts. However, data from the *ICSBP*-transfected K-562 cells make a direct effect on *bcr-abl* unlikely. Thus, the possible interaction between *ICSBP* and transforming genes need to be determined, especially a possible effect of *bcr-abl* on *ICSBP* expression. In addition, the 'homing' of myeloid cells could be negatively influenced by the lack of *ICSBP,* ie, through the loss of expression of certain adhesion molecules. Interestingly, IFN-α therapy may induce adhesion molecules, and this, at least in part, may explain its beneficial effect in human CML.[31] On the other hand, IFN-α induces negative cytokines in the human bone marrow stromal cells, and this has been implicated in the therapeutic efficacy of these drugs in treating CML patients.[32,33]

In summary, *ICSBP* loss induces myeloid leukemias in mice, and this report describes that *ICSBP*-mRNA is very low or absent in the majority of human myeloid leukemias. Together these data raise the possibility that *ICSBP* may be a tumor-suppressor, but the exact role *ICSBP* plays in hematopoiesis remains to be determined.

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REFERENCES

1. Petska S, Langer JA, Zoon KC, Samuel CE: Interferons and their actions. Annu Rev Biochem 56:727, 1987

2. Williams BRG: Transcriptional regulation of interferon-stimulated genes. Eur J Biochem 200:1, 1991

3. Tanaka N, Taniguchi T: Cytokine gene regulation: Regulatory cis elements and DNA binding factors involved in the interferon system. Adv Immunol 52:263, 1992

4. Driggers PH, Ennist DL, Gleason SL, Mak W-H, Marks MS, Levi B-Z, Flanagan JR, Appella E, Ozato K: An interferon γ-regulated protein that binds the interferon-inducible enhancer-element of major histocompatibility complex class I genes. Proc Natl Acad Sci USA 87:3743, 1990

5. Weisz A, Marx P, Sharf R, Appella E, Driggers PH, Ozato K, Levi B-Z: Human interferon consensus sequence binding protein is a negative regulator of enhancer elements common to interferon-inducible genes. J Biol Chem 267:25589, 1992

6. Miyamoto M, Fujita T, Kimura Y, Maruyama M, Harada H, Sudo Y, Miyata T, Taniguchi T: Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. Cell 54:903, 1988

7. Harada H, Fujita T, Miyamoto M, Kimura Y, Maruyama M, Furia A, Miyata T, Taniguchi T: Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell 58:729, 1989

8. Au W-C, Moore PA, Lowther W, Juang Y-T, Pitha PM: Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. Proc Natl Acad Sci USA 92:11657, 1995

9. Veals SA, Schindler C, Leonard D, Fu X-Y, Aebersold R, Darnell JE Jr, Levy DE: Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins. Mol Cell Biol 12:3315, 1992

10. Yamagata T, Nishida J, Tanaka T, Sakai R, Mitani K, Yoshida M, Taniguchi T, Yazaki Y, Hirai H: A novel interferon regulatory factor family transcription factor, ICSAT/Pip/LSIRF, that negatively regulates the activity of interferon-regulated genes. Mol Cell Biol 16:1283, 1996

11. Nelson N, Kanno Y, Hong C, Contursi C, Fujita T, Fowlkes BJ, O'Connel E, Hu-Li J, Paul WE, Jankovic D, Sher AF, Coligan JE, Thornton A, Appella E, Yang Y, Ozato K: Expression of interferon regulatory factor family proteins in lymphocytes. Induction of Stat 1 and *ICSBP* expression by T cell activation. J Immunol 156:3711, 1996

12. Matsuyama T, Kimura T, Kitagawa M, Pfeffer K, Kawakami T, Watanabe N, Kündig TM, Amakawa R, Kishihara K, Wakeham A, Potter J, Furlonger CL, Naredran A, Suzuki H, Ohashi PS, Paige CJ, Taniguchi T, Mak TW: Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. Cell 75:83, 1993

13. Willman CL, Sever CE, Pallavicini MG, Harada H, Tanaka N, Slovak ML, Yamamoto H, Harada K, Meeker TC, List AF, Taniguchi T: Deletion of IRF-1, mapping to chromosome 5q31.1, in human leukemia and preleukemic myelodysplasia. Science 259:968, 1993

14. Nagarajan L, Zavadie J, Claxton D, Lu X, Fairman J, Warrington JA, Wasmuth JJ: Consistent loss of the D5S89 locus mapping telomeric to the interleukin gene cluster and centromeric to EGR-1 in patients with 5q (-) chromosome. Blood 83:199, 1994

15. Fischer T, Aman J, van der Kuip H, Rudolf G, Peschel C, Aulitzky WE, Huber C: Induction of interferon regulatory factors, 2′-5′ oligoadenylate synthetase, P68 kinase and RNase L in chronic myelogenous leukemia cells and its relationship to clinical responsiveness. Br J Haematol 92:595, 1996

16. Holtschke T, Löhler J, Kanno Y, Fehr T, Giese N, Rosenbauer F, Lou J, Knobeloch K-P, Gabriele L, Waring JF, Bachmann MF, Zinkernagel RM, Morse HC III, Ozato K, Horak I: Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the *ICSBP* gene. Cell 87:307, 1996

17. Nowell PC, Hungerford DA: A minute chromosome in human chronic granulocytic leukemia. Science 132:1497, 1960

18. Rowley JD: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa banding. Nature 243:290, 1973

19. Seelig R, Schranz P, Bottner C, Neubauer A, Seelig HP, Renz M: The chronic myelocytic cell line K 562 contains minor (m) as well as major (M) bcr/abl fusion mRNAs. Leukemia 7:1886, 1993

20. Nagel S, Schmidt M, Thiede C, Huhn D, Neubauer A: Quantification of Bcr-Abl transcripts in chronic myelogenous leukemia (CML) using standardized, internally controlled, competitive differential PCR (CD-PCR). Nucleic Acids Res 24:4102, 1996

21. Talpaz M, Kantarjian HM, McCredie K, Truillo JM, Keating MJ, Gutterman JU: Hematologic remission and cytogenetic improvement induced by recombinant human interferon alpha(A) in chronic myelogenous leukemia. N Engl J Med 314:1065, 1986

22. Tura S, Baccarani M, Zuffa E, Russo D, Fanin R, Zaccaria A, Fiacchim M: Interferon alfa-2a as compared with conventional chemotherapy for the treatment of chronic myeloid leukemia. N Engl J Med 330:820, 1994

23. Allan NC, Richards SM, Shepherd PCA: UK Medical Research Council randomized multicentre trial of interferon-alpha n1 for chronic myeloid leukemia: Improved survival irrespective of cytogenetic response. Lancet 345:1392, 1995

24. Talpaz M, Chernajovsky Y, Troutman-Worden K, Wetzler M, Kantarjian H, Gutterman JU, Kurzrock R: Interferon-stimulated genes in interferon-sensitive and resistant chronic myelogenous leukemia patients. Cancer Res 52:1087, 1992

25. Otterson GA, Khleif SN, Chen W, Coxon AB, Kaye FJ: CDKN2 gene silencing in lung cancer by DNA hypermethylation and kinetics of p16INK4 protein induction by 5-aza 2′deoxycytidine. Oncogene 11: 1211, 1995

26. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidrausky D: 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1:686, 1995

27. Kurzrock R, Talpaz M, Kantarjian H, Walters R, Saks S, Trujillo JM, Gutterman JU: Therapy of chronic myelogenous leukemia with recombinant interferon-γ. Blood 70:943, 1987

28. Kloke O, May D, Wandl U, Becher R, Opalka B, Beer U, Niederle N: Treatment of chronic myelogenous leukemia with interferons alpha and gamma. Blut 61:45, 1990

29. Russo D, Fanin R, Zuffa E, Damiani D, Gallizia C, Michieli M, Testoni N, Benfenati D, Visani G, Zaccaria A, Baccarani M, Tura S: Sequential treatment of Ph-positive chronic myeloid leukemia with interferon gamma and interferon alpha. Haematologica 75:334, 1990

30. Opalka B, Wandl UB, Becher R, Kloke O, Nagel-Hiemke M, Moritz T, Beer U, Seeber S, Niederle N: Minimal residual disease in patients with chronic myelogenous leukemia undergoing long-term treatment with recombinant interferon alpha-2b alone or in combination with interferon gamma. Blood 78:2188, 1991

31. Bhatia R, Wayner EA, McGlave PB, Verfaillie CM: Interferon-alpha restores normal adhesion of chronic myelogenous leukemia hematopoietic progenitors to bone marrow stroma by correcting impaired beta 1 integrin receptor function. J Clin Invest 94:384, 1994

32. Aman MJ, Rudolf G, Goldschmitt J, Aulitzky WE, Lam C, Huber C, Peschel C: Type-I interferons are potent inhibitors of interleukin-8 production in hematopoietic and bone marrow stromal cells. Blood 82:2371, 1993

33. Aman MJ, Keller U, Derigs G, Mohamadzadeh M, Huber C, Peschel C: Regulation of cytokine expression by interferon-alpha in human bone marrow stromal cells: Inhibition of hematopoietic growth factors and induction of interleukin-1 receptor antagonist. Blood 84:4142, 1994