Increase of T and B cells and altered BACH2 expression patterns in bone marrow trephines of imatinib-treated patients with chronic myelogenous leukaemia

MAXIMILIAN VON LAFFERT[1], MATHIAS HÄNEL[2], MANFRED DIETEL[1],
IOANNIS ANAGNOSTOPOULOS[1] and KORINNA JÖHRENS[1]

[1]Institute of Pathology, Charité Universitätsmedizin, D-10117 Berlin;
[2]Department of Internal Medicine III, Klinikum Chemnitz gGmbH, D-09113 Chemnitz, Germany

Abstract. The effect of imatinib on T and B cells in patients with chronic myelogenous leukaemia (CML) is not well understood. An upregulation of the transcription factor Broad-complex-Tramtrack-Bric-a-Brac and Cap'n'collar 1 bZip transcription factor 2 (BACH2), which is involved in the development and differentiation of B cells, was demonstrated in a CML cell line treated with imatinib. The present study retrospectively analysed the expression and distribution of cluster of differentiation (CD)3, CD20 and BACH2 (per 1,000 cells), as well as the co-expression of CD20 and BACH2, using immunohistochemistry in serial bone marrow trephines obtained from 14 CML patients treated with imatinib in comparison to 17 patients with newly diagnosed CML and 6 control trephines. Bone marrow trephines of CML patients in remission under imatinib therapy exhibited significantly higher numbers of CD3 and CD20 infiltrates (partly ordered in aggregates) compared with patients with newly diagnosed CML and control individuals. Similarly, nuclear expression of BACH2 in granulopoietic cells was increased in CML patients treated with imatinib, which may represent the histological correlate of the positive treatment effect. Furthermore, since BACH2 is involved in B cell development, its altered expression patterns by imatinib may be one explanation for high B cell numbers, as revealed by CD20/BACH2 (nuclear)-positive cells. As the present data are preliminary, future prospective studies are required to assess the prognostic and predictive role of BACH2 in patients with CML under targeted therapy.

Correspondence to: Dr Maximilian von Laffert, Institute of Pathology, Charité Universitätsmedizin, Charitéplatz 1, D-10117 Berlin, Germany
E-mail: maximilian.von-laffert@charite.de

Introduction

The tyrosine kinase inhibitor (TKI) imatinib, also known as STI571, Glivec® and Gleevec®, which is also active against B cell receptor/Abelson murine leukemia viral oncogene homolog 1 (BCR/ABL1) kinase, has been approved as a first line therapy for Philadelphia-chromosome positive chronic myelogenous leukaemia (CML) with good therapy response data (1-5). Even though the drug is well tolerated, it exhibits an effect on the immune system that is presently not well understood, but may possibly be due to a modulation of various T and B cell response mechanisms (4,6-14). As bone marrow trephines are performed during TKI-treatment, identifying the possible effects of treatment on the number, distribution and immunophenotype of lymphocytes is important for assessing these cases.

It is notable that BCR-ABL1 was demonstrated to repress Broad-complex-Tramtrack-Bric-a-Brac and Cap'n'collar 1 bZip transcription factor 2 (BACH2), a transcription factor involved in the development and differentiation of B cells, whereas cluster of differentiation (CD)34+ cells treated *in vitro* with imatinib exhibited a consistent mRNA accumulation and nuclear repositioning of BACH2 (15,16). The BACH family of transcription factors, which mediate transcriptional repression, comprises BACH1 and BACH2. Whereas BACH1 is expressed ubiquitously, BACH2 (mapped to chromosome 6q15) is primarily observed in neuronal cells and during specific stages of B cell differentiation (17-19). In particular, BACH2 is central in the late stages of B cell development and is a critical component of the transcription factor network, which regulates the terminal differentiation of mature B cells into antibody-secreting plasma cells. During this, BACH2 is required to delay the plasma cell differentiation long enough to allow class switch recombination and somatic hypermutation (17,20-23).

Overall, it may be hypothesised that upregulation of BACH2 via blockage of BCR/ABL1 tyrosine kinase plays an immunomodulatory role, which affects bone marrow lymphocytes. Previous aforementioned studies refer to either serum, bone marrow aspirates, cell cultures or mouse models; however, data obtained from human bone marrow trephines are scarce. Therefore, the present study retrospectively investigated bone marrow trephines of patients with newly diagnosed

CML, and CML patients in remission under imatinib treatment for the presence and distribution of T and B cells, as well as for BACH2 expression patterns.

Patients and methods

Patients. For the present retrospective analysis, a total of 53 paraffin-embedded bone marrow trephines were obtained between October 2006 and October 2014 from 37 patients from the archive of the Institute of Pathology, Charité Universitätsmedizin (Berlin, Germany). The trephines were from the following patient groups: CML group (initial diagnosis), follow-up CML group (under imatinib therapy) and control group.

The CML group comprised of 17 trephines obtained from 17 patients at the time of initial CML diagnosis (4 women; 13 men; mean age at diagnosis, 58.4 years; age range at diagnosis, 23-74 years). CML diagnosis was made according to the World Health Organisation (WHO) guidelines (24), including the detection of BCR/ABL1-fusion by polymerase chain reaction and/or fluorescence *in situ* hybridization. No follow-up data were available for these patients.

The follow-up CML group comprised of 30 bone marrow trephines from 14 patients with an established diagnosis of CML (4 women; 10 men; mean age at diagnosis, 46.7 years; age range at diagnosis, 5-73 years). Diagnosis was made at the Department of Internal Medicine III, Klinikum Chemnitz gGMBH (Chemnitz, Germany), according to the WHO guidelines (24). At least two follow-up trephines per patient were available, obtained at various time points during treatment with imatinib (range, 3-104 months). Therapy response was classified as complete molecular remission (CMR), major molecular remission (MMR) and complete cytogenetic remission (CCgR). For two patients (1 and 8) these data were not available; since their bone marrow trephines did not exhibit any CML relapse; therefore, they were classified as haematological remission (HR).

The control group comprised of 6 bone marrow trephines from 6 patients, who underwent investigation to exclude a disease of the haematolymphoid system (2 women; 4 men; mean age at the time of biopsy, 50.5 years; age range at time of biopsy, 26-71 years). In all cases, the bone marrow was normocellular without any signs of haematolymphoid malignancy.

All 53 samples were anonymised. Dr Korinna Jöhrens and Dr Maximilian von Laffert (Institute of Pathology, Charité Universitätsmedizin) performed histomorphological and immunohistological evaluation without knowledge of diagnosis and therapy duration. The present study was conducted according to institutional guidelines, and was approved by the Ethics Commission of the Charité Universitätsmedizin (approval no., EA4/129/15). Detailed morphological descriptions of all 53 trephines with the corresponding clinical data, including age, gender, interval of biopsy following CML-diagnosis and remission status, are shown in Tables I-III.

Histology and immunohistochemistry. All bone marrow trephines were fixed in 4% formaldehyde, decalcified using an EDTA-containing solution and embedded in paraffin. Sections were cut to 4 μm-thick and stained with haematoxylin and eosin, Giemsa, Gomori silver impregnation, iron and the periodic acid-Schiff reaction. Deparaffinisation, as well as epitope retrieval and immunostainings, were performed within the automated Leica Bond-Max™ System (Leica Biosystems GmbH, Nussloch, Germany). The following primary antibodies were used: Mouse monoclonal anti-glycophorin C (clone, Ret40f; catalog no., M0820; dilution, 1:400), CD20 (clone, L26; catalog no., M0755; dilution, 1:750) and CD68 (clone, PG-M1; catalog no., M0876; dilution, 1:200) (Dako, Glostrup, Denmark); mouse monoclonal anti-CD34 (clone, QBEnd/10; catalog no., END-L-CE; dilution, 1:50) and CD3 (clone, LN10; catalog no., CD3-565-L-CE; dilution, 1:50) (Novocastra™, Leica Biosystems, Ltd., Newcastle upon Tyne, UK); mouse monoclonal anti-CD61 (clone, 2f2; catalog no., PA0308; dilution, 1:50; Leica Biosystems, Ltd.); rabbit polyclonal anti-myeloperoxidase (catalog no., RB-373-A; dilution, 1:300; Thermo Fisher Scientific, Inc., Waltham, MA, USA); and rabbit polyclonal anti-BACH2 (catalog no., LS-B3533; dilution, 1:200; LifeSpan Biosciences, Eching, Germany). For visualization of bound antibodies, Bond Polymer Refine Diaminobenzidine (DAB) kit was used (Leica Biosystems, Ltd). In order to verify BACH2 expression in B cells, a double immunostaining protocol was used, as available in the Bond-Max™ System. BACH2 expression was visualized using the Bond Polymer Alkaline Phosphatase kit, resulting in a red stain, while CD20 was visualized with the Bond Polymer Refine DAB kit, resulting in a brown stain.

Distribution and quantification of lymphocytes and BACH2 expression. In all samples, the distribution of T and B cells was investigated and classified as interstitial or para-trabecular. Compact cell clusters, forming a cohesive unit of ≥50 lymphocytes, were defined as aggregates. Solitary or loosely distributed lymphocytes were defined as infiltrates. BACH2 expression was classified as nuclear (nuc) or cytoplasmic (cp). In each trephine, 1,000 cells were counted, and the expression of CD3, CD20 and BACH2 was evaluated, as well as the co-expression of the latter two. The 1,000 cells viewed per trephine consisted of 10 representative fields, each with 100 cells. The aforementioned aggregates were evaluated separately and were not part of the 1,000 cell counting process. In each aggregate, the total number of CD3, CD20 and CD20/BACH2 staining cells was counted. Counting and evaluation was performed at x40 and x60 objective with a 25 mm field of view ocular (BX40, Olympus Europa Holding GmbH, Hamburg, Germany). Two pathologists (Dr Maximilian von Laffert and Dr Korinna Jöhrens) evaluated the stainings independently.

Statistical analysis. Shapiro-Wilk test was used to test if the parameters [CD3, CD20, CD20/BACH2 (nuc), BACH2 (nuc), BACH2 (cp)] within the three groups (CML, follow-up CML and control groups) followed a normal distribution; $P<0.05$ indicated that parameters were not normally distributed. Kruskal-Wallis test was used to evaluate if the distribution of the parameters [CD3, CD20, CD20/BACH2 (nuc), BACH2 (nuc), BACH2 (cp)] was identical over the three groups (null hypothesis; H0). H0 was rejected for $P<0.05$; H1 indicated a significant difference between the three groups. Statistical analysis was performed using SPSS version 21 software (IBM SPSS, Armonk, NY, USA).

Table I. Follow-up CML group. Detailed description of morphological findings from 30 biopsies at different time points of 14 patients with CML under imatinib therapy.

Case no.	Age	Gender	No. of biopsies	Time of biopsy, months[a]	Remission status	Staining per 1,000 cells					Aggregate cell total
						CD3	CD20	BACH2 (cp)	BACH2 (nuc)	CD20:BACH2 (nuc)	CD3:CD20:CD20:BACH2 (nuc)
1	73	F	2	12	HR	202	120	100	9	40	-
				15		223	150	100	21	100	20:30:22 and 12:21:11
2	10	M	2	12	MMR	185	163	100	108	69	-
				18		220	128	100	115	51	33:19:15 and 35:21:15
3	54	M	3	3	CCgR	195	152	300	132	120	10:22:21
				6		150	75	100	10	15	-
				9		223	109	100	12	104	15:51:30
4	68	M	3	3	MMR/CCgR	288	71	100	60	35	18:38:6
				6		121	63	250	30	20	-
				11		344	68	100	41	30	55:82:50 and 35:52:30
5	65	F	2	102	MMR/CCgR	195	96	350	75	45	53:51:40
				104		181	94	100	17	80	25:65:20
6	10	M	2	23	CMR	226	187	100	123	150	33:32:25
				26		343	62	100	5	28	-
7	49	M	2	60	CMR	280	98	600	6	30	53:72:21
				70		224	135	100	12	87	49:52:13
8	5	F	2	6	HR	325	248	100	269	202	-
				12		180	47	100	38	35	-
9	23	M	2	12	CMR/CCgR	186	52	350	8	13	-
				24		389	75	100	22	62	25:26:15
10	43	M	2	48	CMR	256	121	200	20	100	55:126:20
				64		134	110	100	31	80	-
11	48	F	2	12	CMR/CCgR	452	138	300	2	110	51:55:10, 30:28:3, 45:73:8 and 23:24:5
				60		359	73	600	5	4	51:65:8, 38:42:2 and 25:31:2
12	48	M	2	54	CMR/CCgR	334	87	600	2	20	35:51:18 and 52:65:28
				60		464	138	350	3	24	59:68:9, 60:58:8, 37:38:5 and 28:25:2
13	67	M	2	3	CMR/CCgR	625	150	400	35	70	-
				36		428	240	400	24	100	45:38:10
14	51	M	2	12	CMR/CCgR	461	133	400	32	39	-
				35		354	115	600	25	38	-

Cell numbers for CD3, CD20, BACH2 (cp), BACH2 (nuc), as well as CD20/BACH2 (nuc) are given per 1,000 cells and reflect the defined infiltrates. If aggregates were identified (n=30), the absolute cell counts for CD3, CD20 and CD20/BACH2 (nuc) are provided. [a]Time of biopsy following CML diagnosis. CML, chronic myelogenous leukaemia; CD, cluster of differentiation; BACH2, Broad-complex–Tramtrack–Bric-a-Brac and Cap'n'collar 1 bZip transcription factor 2; cp, cytoplasmic; nuc, nuclear. HR, haematologic remission; MMR, major molecular remission; CCgR, cytogenetic remission; CMR, complete molecular remission.

Table II. CML inital diagnosis group. Detailed description of the morphological findings of 17 biopsies of 17 patients with CML at initial diagnosis.

| Case no. | Age | Gender | Staining per 1,000 cells | | | | | Aggregate cell total |
			CD3	CD20	BACH2 (cp)	BACH2 (nuc)	CD20/BACH2 (nuc)	CD3:CD20:CD20/BACH2 (nuc)
1	48	M	75	19	556	5	7	-
2	72	F	56	12	423	4	8	-
3	74	M	49	13	380	1	10	-
4	66	M	41	2	440	1	1	-
5	72	M	53	18	360	6	13	-
6	55	M	73	15	448	1	10	-
7	63	M	51	1	423	0	1	-
8	67	F	62	6	658	5	4	-
9	58	M	58	9	758	10	6	-
10	40	M	44	10	776	11	7	-
11	59	F	51	8	860	6	5	-
12	69	F	54	2	590	1	2	-
13	42	M	57	3	660	3	2	-
14	45	M	56	7	550	3	3	68:30:25 and 42:26:2
15	64	M	51	6	440	1	4	38:109:25
16	23	M	45	2	767	1	2	-
17	74	M	57	19	667	0	8	42:35:10 and 32:14:7

Cell numbers for CD3, CD20, BACH2 (cp), BACH2 (nuc), as well as CD20/BACH2 (nuc) are given per 1,000 cells and reflect the defined infiltrates. If aggregates were identified (n=5), the absolute cell counts for CD3, CD20 and CD20/BACH2 (nuc) are provided. CML, chronic myelogenous leukaemia; CD, cluster of differentiation; BACH2, Broad-complex-Tramtrack-Bric-a-Brac and Cap'n'collar 1 bZip transcription factor 2; cp, cytoplasmic; nuc, nuclear.

Table III. Control group. Detailed description of the morphological findings of 6 biopsies of 6 patients with benign bone marrow.

| Case no. | Age | Gender | Staining per 1,000 cells | | | | | Aggregate cell total |
			CD3	CD20	BACH2 (cp)	BACH2 (nuc)	CD20/BACH2 (nuc)	CD3:CD20:CD20/BACH2 (nuc)
1	45	F	135	96	510	25	87	-
2	71	M	168	35	195	11	10	25:62:10
3	30	F	146	41	146	28	18	-
4	26	M	105	39	304	35	12	-
5	67	M	111	20	197	20	9	41:26:3
6	64	M	159	19	396	8	7	-

Cell numbers for CD3, CD20, BACH2 (cp), BACH2 (nuc), as well as CD20/BACH2 (nuc) are given per 1,000 cells and reflect the defined infiltrates. If aggregates were identified (n=2), the absolute cell counts for CD3, CD20 and CD20/BACH2 (nuc) are provided. CML, chronic myelogenous leukaemia; CD, cluster of differentiation; BACH2, Broad-complex-Tramtrack-Bric-a-Brac and Cap'n'collar 1 bZip transcription factor 2; cp, cytoplasmic; nuc, nuclear.

Results

Lymphocytic aggregates and infiltrates. Compared to the CML (initial diagnosis) and control groups, the follow-up CML group (under therapy) exhibited an increased amount of interstitial lymphocytic aggregates and infiltrates; the latter being either solitary or loosely distributed (Tables IV and V).

In particular, aggregates were observed in 60% (18/30) of the trephines [86% (12/14) of the patients] in the follow-up CML group. A total of 7 trephines exhibited >1 aggregate (maximum, 4 aggregates per trephine), and a total of 30 aggregates were identified with B cells slightly outnumbering T cells (mean ratio of CD3:CD20, 36.8:47.4). In comparison, 5 aggregates were identified in the 17 trephines of the CML group at

Table IV. Total number of aggregates and their mean immunohistochemical composition within the three different groups.

Group	No. of biopsies	Total number of aggregates (range per trephine)	Mean composition of aggregates CD3:CD20:CD20/BACH2 (nuc)
CML (initial diagnosis)	17	5 (0-2)	44.4:42.8:13.8
Follow-up CML (under therapy)	30	30 (0-4)	36.8:47.4:15.7
Control	6	2 (0-1)	33.0:44.0:6.5

For a detailed immunohistochemical composition of each infiltrate see Tables I-III. CML, chronic myelogenous leukaemia; CD, cluster of differentiation; BACH2, Broad-complex-Tramtrack-Bric-a-Brac and Cap'n'collar 1 bZip transcription factor 2; nuc, nuclear.

Table V. Mean immunohistochemical compositions of lymphoid infiltrates per 1,000 cells within the three different groups.

Staining	Mean CML diagnosis group (range)	Mean follow-up CML group (range)	Mean control group (range)	P-value
CD3	54.9 (41-75)	284.9 (121-625)	137.3 (105-168)	<0.05
CD20	8.9 (1-19)	116.6 (47-248)	41.7 (19-96)	<0.05
BACH2 (cp)	573.9 (360-860)	243.3 (100-600)	291.3 (146-510)	<0.05
BACH2 (nuc)	3.5 (0-11)	43.1 (2-269)	21.2 (8-35)	<0.05
CD20/BACH2 (nuc)	5.5 (1-13)	63.4 (4-202)	23.8 (7-87)	<0.05

For a detailed immunohistochemical composition of each infiltrate see Tables I-III. CML, chronic myelogenous leukaemia; CD, cluster of differentiation; BACH2, Broad-complex-Tramtrack-Bric-a-Brac and Cap'n'collar 1 bZip transcription factor 2; cp, cytoplasmic; nuc, nuclear.

diagnosis (mean ratio of CD3:CD20, 44.4:42.8) and 2 in the control group (mean ratio of CD3:CD20, 33:44) (Tables I-IV).

Compared to the CML and the control group, the follow-up group exhibit a higher amount of solitary and/or loosely distributed lymphocytes consisting of CD3- and CD20-positive cells. These types of infiltrates were observed in all follow-up group trephines, with T cells outnumbering B cells. In more detail, within the 17 trephines with CML the mean value for CD3 and CD20 per 1,000 cells was 54.9 (range, 41-75) and 8.9 (range, 1-19), respectively, compared with 284.9 (range: 121-625) and 116.6 (range, 47-248) for CD3 and CD20, respectively, for the follow-up group under therapy. The control group had mean values of 137.3 (range, 105-168) for CD3 and 41.7 (range, 19-96) for CD20 per 1,000 cells (Tables I-III and V).

BACH2 expression and CD20/BACH2 co-expression patterns. BACH2 expression was observed in the cp and nuc of the granulopoietic cells. Furthermore, an expression of BACH2 (nuc) was identified in the above-described infiltrates and aggregates rich in CD20 cells. For an improved understanding of these BACH2 (nuc)-positive cells additional CD20/BACH2 double stainings were performed in all 53 trephines.

In the initial CML diagnosis group, the mean value in the granulopoietic cells for BACH2 (cp) was 573.9 per 1,000 cells (range, 360-860) and 3.5 (range, 0-11) for BACH2 (nuc). These values differed from the two other groups, which revealed a mean expression of 243.3 (range, 100-600) for BACH2 (cp) and 43.1 (range, 2-269) for BACH2 (nuc) in the follow-up group under treatment with imatinib, and mean values of 291.3 (range, 146-510) for BACH2 (cp) and 21.2 (range, 8-35) for BACH2 (nuc) in the control group. Evaluation of double

staining in the solitary and loosely distributed lymphocytes revealed that the mean value for CD20/BACH2 (nuc) was 5.5 per 1,000 cells (range, 1-13) for samples in the CML initial diagnosis group, 63.4 (range, 4-202) in the follow-up treatment group and 23.8 (range, 7-87) in the control group (Fig. 1A-C; Tables I-III and V). Within the lymphocytic aggregates a subpopulation of B cells also exhibited nuclear BACH2 expression in all three groups (Fig. 1D; Tables I-IV).

Statistical analysis, association with remission status and other clinical factors. Within the analyzed groups (CML, follow-up and control), the five parameters [CD3, CD20, BACH2 (cp), BACH2 (nuc) and CD20/BACH2 (nuc)] did not follow a normal distribution (P<0.05) and were significantly different (P<0.05) as tested by Kruskal-Wallis (Table V). In detail, differences between the three groups were significant and revealed higher amounts of T and B cells in CML-patients treated with imatininb compared to patients in the initial CML diagnosis and control groups. The same was true for BACH2 (nuc) expressions in granulopoietic cells and B cells, whereas BACH2 (cp) was higher in the granulopoietic cells of the inital diagnosis CML group.

Following anonymised analysis of all samples, the follow-up group was associated with available clinical data, particularly concerning therapy response/remission status, as well as duration of treatment, and alterations in health status, including acute infection and therapy regimen (data from electronic health records). At the time of biopsy (follow-up time between 9 and 104 months), all patients exhibited therapy responses (Table I). The remission status for patients in the follow-up CML group was as follows: 8 patients with CMR,
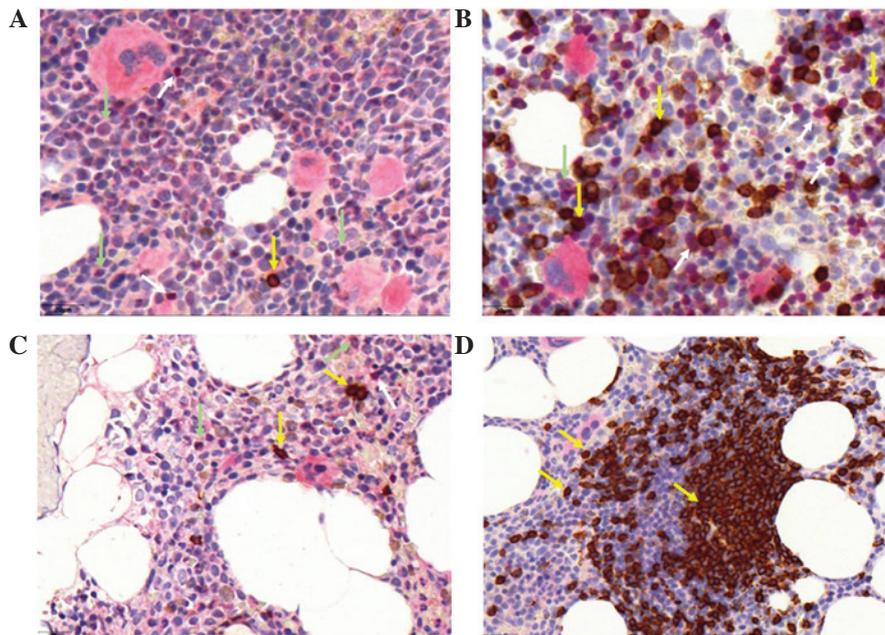
Figure 1. CD20/BACH2 double immunostains in bone marrow trephines of patients in CML at diagnosis, CML under imatinib treatment and control groups. (A) Bone marrow in patients with CML at initial diagnosis contained a low number of CD20-positive B cells, with additional BACH2 expression. Granulopoietic cells exhibited a clear cytoplasmic and focal nuclear expression of BACH2. (B) Patients with CML under imatinib treatment had bone marrow that exhibited an increased number of granulopoietic cells with nuclear BACH2 expression, and an increased number of CD20-positive B cells, partly with nuclear BACH2 expression. (C) Bone marrow from patients in the control group contained higher numbers of B cells coexpressing BACH2 and granulopoietic cells with nuclear BACH2 expression compared with CML at diagnosis, but lower numbers of both cell types compared with CML under imatinib treatment. (D) Increased B cells under imatinib treatment also formed aggregates that contained T cells. Certain B cells within these aggregates exhibited nuclear BACH2 expression. Yellow arrows, co-expression of CD20/BACH2; white arrows, nuclear BACH2 expression in granulopoietic cells; green arrows, cytoplasmic BACH2 expression in granulopoietic cells. CML, chronic myelogenous leukaemia; CD, cluster of differentiation; BACH2, Broad-complex-Tramtrack-Bric-a-Brac and Cap'n'collar 1 bZip transcription factor 2.

3 patients with MMR, 1 patients with CCgR and 2 patients with HR. As aforementioned, patients with initially diagnosed CML exhibited a maximum of 11 cells per 1,000 with nuclear BACH2 expression (mean, 3.5; range, 0-11), the control group exhibited nuclear BACH2 expression between 8 and 35 cells per 1,000 (mean, 21.2), while the mean expression in the CML follow-up treatment group was 43.1 (range, 2-269). Therefore, it appears that a nuclear BACH2 cut off of >11 for therapy response may be appropriate in the present collective.

No association was detectable between BACH2 expression and duration of imatinib treatment (range, 3-104 months). Additional analysis of the clinical data revealed no affect on the health status or other disorders, including acute infections or an alteration to the therapeutic regimen, at time of biopsy.

Discussion

Cytological and histological investigation of peripheral blood and bone marrow with consecutive cytogenetic demonstration of the Philadelphia chromosome, and/or the molecular detection of BCR/ABL1 transcript, forms the diagnostic basis of CML and leads to consecutive targeted therapy (24-26). Although therapy response is encouraging, the consequences on the immune system, due to ABL tyrosine kinase blockage, are far away from being well understood and are discussed controversially (6-14). Furthermore, knowledge of possible histomorphological alterations are important for the evaluation of bone marrow trephines obtained during TKI treatment.

The present retrospective analysis of serial bone marrow trephines from CML patients in various stages of haematologic, cytogenetic and molecular remission under imatinib therapy (Table I) revealed significantly higher numbers of CD3- and CD20-positive infiltrating cells (partly ordered in aggregates) compared with trephines of CML patients at the time of diagnosis and control trephines. Similar results were obtained for the nuclear expression of BACH2 in granulopoietic cells, whereas the amount of cytoplasmic BACH2 accumulation was lower. These BACH2 expression shifts may represent the histological correlate of the positive effects of the treatment strategy. Furthermore, this induced BACH2-stimulation may lead to additional B cell differentiation, which is demonstrated by the occurrence of CD20/BACH2 (nuc)-positive cells. The number of increased T cells in the imatinib-treated patient group is in line with findings observed under dasatinib treatment, where large granular lymphocytes and natural killer cells were revealed to play a role in CML immune surveillance (27,28). Similar observations have been reported in imatinib-treated Philadelphia chromosome positive B lymphoblastic leukaemia (29), demonstrating a predominant presence of CD4 and CD8 lymphocytes directed against the p190 BCR/ABL protein in bone marrow in comparison to peripheral blood. The occurrence of such cells has been demonstrated to be associated with lower minimal residual disease values, whereas their absence is reported in relapse patients (29).

The high CD20 counts observed in the present follow-up CML patients under imatinib therapy (Fig. 1B and D; Table V) are similar to the flow cytometry data identified by

Rohon *et al* (30), which revealed lower proportions of B cells and dendritic cells at the time of CML diagnosis and that these levels increase (normalize) during TKI-therapy in bone marrow and peripheral blood. In addition, the present study identified a frequent nuc expression of BACH2 in granulopoietic cells and in a proportion of B cells, whereas BACH2 nuc expression in granulopoietic cells and B cells was significantly lower in trephines from CML initial diagnosis and control groups (Table V). Regarding BACH2 in the context of CML, the BCR/ABL1 fusion protein leads, among other pathways, to an increased phosphorylation and nuclear export of BACH2. This results in an enhanced cell survival owing to resistance to oxidative stress (17,31). This is reflected by the present histological examination, which revealed a low amount of nuc, but a high amount of cp BACH2 intensity in the granulopoietic cells of newly diagnosed CML patients (Fig. 1A; Table V). The observation of nuclear BACH2 expression in granulopoietic cells in the imatinib-treated CML patients (Fig. 1B) presents, to the best of our knowledge, the first *in vivo* demonstration of a finding previously reported in cultured BCR/ABL1 positive cells (15,16). This accumulation and nuclear repositioning, or translocation to the nucleus, of BACH2 obviously increases the rate of apoptosis, and thus enhances the cytotoxic effect of therapy, as demonstrated by previous studies (15-17,32). This type of nuclear translocation was also discussed as a modulator of the therapy response in mantle cell lymphoma (33). Whereas nuclear BACH2 expression is reduced in CML, imatinib leads to normalization and/or an increase in nuclear BACH2 levels. Therefore, at first sight, a nuclear BACH2 cut off of >11 for therapy response (normalization of the BACH2 level) may be appropriate in the present cohort. However, there were two patients in the responder group that exhibited an expression of <11 per 1,000 cells in their trephines. Therefore, it appears to be important to regard the therapy response data in the context of the development of BACH2 expressions compared with the baseline (CML diagnosis) and a parallel increase of T and B cells. Thus, the present data are preliminary and to identify a distinct cut off, a larger and comparative study is required.

Furthermore, as the present study reveals, the imatinib-associated BACH2 upregulation may lead to its nuc expression in a proportion of B cells, and this therefore illustrates one aspect of the complex interplay between BCR/ABL and BACH2, which affects differentiations of lymphoid and myeloid cells. Its role as a prognostic and/or predictive marker in CML, including in the context of imatinib resistance, remains unclear and appears to be worth testing in a prospective clinical context, since its prognostic significance has already been demonstrated in diffuse large B-cell lymphomas (34).

In conclusion, the present study revealed that bone marrow trephines of imatinib-treated CML patients are characterized by an increased number of interstitial lymphoid cells, partly arranged in aggregates. In addition, BACH2 upregulation with nuc expression is observed in granulopoietic and B cells. Therefore, the present study suggests that BACH2 in the context of TKI-receptor blockage has an effect on additional B cell development/differentiation, and its expression pattern may assist in monitoring the response to imatinib at a histological level. As patients of the CML diagnosis group were different to those of the follow-up group, the data reported by the present study requires cautionary interpretation. Future prospective studies are advisable to strengthen the role of BACH2 as a prognostic and predictive marker in CML.

Acknowledgements

References

1. Appel S, Boehmler AM, Grünebach F, Müller MR, Rupf A, Weck MM, Hartmann U, Reichardt VL, Kanz L, Brümmendorf TH and Brossart P: Imatinib mesylate affects the development and function of dendritic cells generated from CD34+ peripheral blood progenitor cells. Blood 103: 538-544, 2004.
2. Appel S, Rupf A, Weck MM, Schoor O, Brümmendorf TH, Weinschenk T, Grünebach F and Brossart P: Effects of imatinib on monocyte-derived dendritic cells are mediated by inhibition of nuclear factor-kappaB and Akt signaling pathways. Clin Cancer Res 11: 1928-1940, 2005.
3. Druker BJ and Lydon NB: Lessons learned from the development of an abl tyrosine kinase inhibitor for chronic myelogenous leukemia. J Clin Invest 105: 3-7, 2000.
4. Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM, Lydon NB, Kantarjian H, Capdeville R, Ohno-Jones S and Sawyers CL: Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 344: 1031-1037, 2001.
5. Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R and Talpaz M: Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med 344: 1038-1042, 2001.
6. Gambacorti-Passerini C, Antolini L, Mahon FX, Guilhot F, Deininger M, Fava C, Nagler A, Della Casa CM, Morra E, Abruzzese E, et al: Multicenter independent assessment of outcomes in chronic myeloid leukemia patients treated with imatinib. J Natl Cancer Inst 103: 553-561, 2011.
7. Dietz AB, Souan L, Knutson GJ, Bulur PA, Litzow MR and Vuk-Pavlovic S: Imatinib mesylate inhibits T cell proliferation in vitro and delayed-type hypersensitivity in vivo. Blood 104: 1094-1099, 2004.
8. Chen J, Schmitt A, Giannopoulos K, Chen B, Rojewski M, Döhner H, Bunjes D and Schmitt M: Imatinib impairs the proliferation and function of CD4+CD25+ regulatory T cells in a dose-dependent manner. Int J Oncol 31: 1133-1159, 2007.
9. Chen J, Schmitt A, Chen B, Rojewski M, Ringhoffer M, von Harsdorf S, Greiner J, Guillaume P, Döhner H, Bunjes D and Schmitt M: Imatinib impairs CD8+ T lymphocytes specifically directed against the leukemia-associated antigen RHAMM/CD168 in vitro. Cancer Immunol Immunother 56: 849-861, 2007.
10. Chen Y, Peng C, Li D and Li S: Molecular and cellular bases of chronic myeloid leukemia. Protein Cell 1: 124-132, 2010.
11. Seggewiss R, Loré K, Greiner E, Magnusson MK, Price DA, Douek DC, Dunbar CE and Wiestner A: Imatinib inhibits T cell receptor-mediated T cell proliferation and activation in a dose-dependent manner. Blood 105: 2473-2479, 2005.
12. Mumprecht S, Matter M, Pavelic V and Ochsenbein AF: Imatinib mesylate selectively impairs expansion of memory cytotoxic T cells without affecting the control of primary viral infections. Blood 108: 3406-3413, 2006.
13. Leder C, Ortler S, Seggewiss R, Einsele H and Wiendl H: Modulation of T effector function by imatinib at the level of cytokine secretion. Exp Hematol 35: 1266-1271, 2007.
14. Catellani S, Pierri I, Gobbi M, Poggi A and Zocchi MR: Imatinib treatment induces CD5+ B lymphocytes and IgM natural antibodies with anti-leukemic reactivity in patients with chronic myelogenous leukemia. PLoS One 6: e18925, 2011.
15. Vieira SA, Deininger MW, Sorour A, Sinclair P, Foroni L, Goldman JM and Melo JV: Transcription factor BACH2 is transcriptionally regulated by the BCR/ABL oncogene. Genes Chromosomes Cancer 32: 353-363, 2001.

16. Ono A, Kono K, Ikebe D, Muto A, Sun J, Kobayashi M, Ueda K, Melo JV, Igarashi K and Tashiro S: Nuclear positioning of the BACH2 gene in BCR-ABL positive leukemic cells. Genes Chromosomes Cancer 46: 67-74, 2007.
17. Rosbrook GO, Stead MA, Carr SB and Wright SC: The structure of the Bach2 POZ-domain dimer reveals an intersubunit disulfide bond. Acta Crystallogr D Biol Crystallogr 68: 26-34, 2012.
18. Oyake T, Itoh K, Motohashi H, Hayashi N, Hoshino H, Nishizawa M, Yamamoto M and Igarashi K: Bach proteins belong to a novel family of BTB basic leucine zipper transcription factors that interact with MafK and regulate transcription through the NF-E2 site. Mol Cell Biol 16: 6083-6095, 1996.
19. Muto A, Hoshino H, Madisen L, Yanai N, Obinata M, Karasuyama H, Hayashi N, Nakauchi H, Yamamoto M, Groudine M and Igarashi K: Identification of Bach2 as a B cell-specific partner for small maf proteins that negatively regulate the immunoglobulin heavy chain gene 3' enhancer. EMBO J 17: 5734-5743, 1998.
20. Igarashi K, Ochiai K and Muto A: Architecture and dynamics of the transcription factor network that regulates B to-plasma cell differentiation. J Biochem 141: 783-789, 2007.
21. Kallies A and Nutt SL: Bach2: Plasma-cell differentiation takes a break. EMBO J 29: 3896-3897, 2010.
22. Nutt SL, Taubenheim N, Hasbold J, Corcoran LM and Hodgkin PD: The genetic network controlling plasma cell differentiation. Semin Immunol 23: 341-349, 2011.
23. Dave SS: The polyphony of BACH2. Blood 123: 950, 2014.
24. Vardiman JW, Melo JV, Baccarani M and Thiele J: Chronic myelogenous leukaemia, BCR-ABL1 positive. In: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J and Vardiman JW (eds). 4th edition. IARC, Lyon, pp32-37, 2008.
25. Reddy EP and Aggarwal AK: The ins and outs of bcr-abl inhibition. Genes Cancer 3: 447-454, 2012.
26. Jabbour E and Kantarjian H: Chronic myeloid leukemia: 2012 update on diagnosis, monitoring and management. Am J Hematol 87: 1038-1045, 2012.
27. Kreutzman A, Juvonen V, Kairisto V, Ekblom M, Stenke L, Seggewiss R, Porkka K and Mustjoki S: Mono/oligoclonal T and NK cells are common in chronic myeloid leukemia patients at diagnosis and expand during dasatinib therapy. Blood 116: 772-782, 2010.
28. Lee SJ, Jung CW, Kim DY, Lee KH, Sohn SK, Kwak JY, Kim HJ, Kim IH, Park S and Kim DH: Retrospective multicenter study on the development of peripheral lymphocytosis following second-line dasatinib therapy for chronic myeloid leukemia. Am J Hematol 86: 346-3450, 2011.
29. Riva G, Luppi M, Barozzi P, Quadrelli C, Basso S, Vallerini D, Zanetti E, Morselli M, Forghieri F, Maccaferri M, *et al*: Emergence of BCR-ABL-specific cytotoxic T cells in the bone marrow of patients with Ph+ acute lymphoblastic leukemia during long-term imatinib mesylate treatment. Blood 115: 1512-1518, 2010.
30. Rohon P, Porkka K and Mustjoki S: Immunoprofiling of patients with chronic myeloid leukemia at diagnosis and during tyrosine kinase inhibitor therapy. Eur J Haematol 85: 387-398, 2010.
31. Yoshida C, Yoshida F, Sears DE, Hart SM, Ikebe D, Muto A, Basu S, Igarashi K and Melo JV: Bcr-Abl signaling through the PI-3/S6 kinase pathway inhibits nuclear translocation of the transcription factor Bach2, which represses the antiapoptotic factor heme oxygenase-1. Blood 109: 1211-1219, 2007.
32. Kamio T, Toki T, Kanezaki R, Sasaki S, Tandai S, Terui K, Ikebe D, Igarashi K and Ito E: B cell-specific transcription factor BACH2 modifies the cytotoxic effects of anticancer drugs. Blood 102: 3317-3322, 2003.
33. Chen Z, Pittman EF, Romaguera J, Fayad L, Wang M, Neelapu SS, McLaughlin P, Kwak L and McCarty N: Nuclear translocation of B Cell-Specific transcription factor, BACH2, modulates ROS mediated cytotoxic responses in mantle cell lymphoma. PLoS One 8: e69126, 2013.
34. Sakane-Ishikawa E, Nakatsuka S, Tomita Y, Fujita S, Nakamichi I, Takakuwa T, Sugiyama H, Fukuhara S, Hino M, Kanamaru A, *et al*: Prognostic significance of BACH2 expression in diffuse large B cell lymphoma: A study of the osaka lymphoma study group. J Clin Oncol 23: 8012-8017, 2005.