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The possible influences of B2A2 and B3A2 BCR/ABL protein structure on thrombopoiesis in chronic myeloid leukaemia

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Abstract

The Philadelphia chromosome, t(9;22)(q34;q11) gives rise more frequently, in chronic myeloid leukaemia (CML), to two *BCR/ABL* chimeric transcripts differing only by the absence of 75 nucleotides and defined as b2a2 and b3a2 types, encoding two 210-kDa tyrosine kinase proteins differing only by the absence of 25 amino acids coded by the b3 exon. In the present study the two transcripts, detected by RT-PCR in 88 consecutive unselected CML patients, were correlated with haematological findings at diagnosis and with the megakaryocyte size and frequency by morphometric evaluation of 45 bone marrow biopsies. The secondary structure prediction and hydrophobicity of the b2a2 and b3a2 type BCR/ABL protein were also obtained. The prediction results for the b3 exon amino acids using GOR IV and NnPredict methods showed a short beta strand corresponding to the hydrophobic portion of the peptide. Significantly higher values were found in the platelet count of patients carrying b3a2 transcripts. The megakaryocyte size and frequency in bone marrow biopsies did not show significant differences between the two groups of patients. Stratifying the patients on the basis of white blood cell (WBC) count below or above 100×10^9/l we still had, in both groups, a significant difference in the platelet count between the b2a2 and b3a2 patients. The possible relationships between the structure of b2a2 and b3a2 types of BCR/ABL fused protein and thrombopoiesis are discussed.

Keywords: Chronic myeloid leukaemia; b2a2 b3a2 mRNA type; BCR/ABL protein structure; Thrombopoiesis; Megakaryocytes

1. Introduction

The Philadelphia (Ph) chromosome, present in chronic myeloid leukaemia (CML) cells, is due to the translocation t(9;22)(q34;q11). At the molecular level this translocation gives rise to a hybrid *BCR/ABL* gene and to the expression of a rearranged mRNA coding in the CML patients for the fusion protein $p210^{bcr/abl}$. In CML, the t(9;22), depending on the localisation of the breakpoint site within the Major breakpoint cluster region (M-bcr) in the *BCR* gene on chromosome 22, gives rise, more frequently, to the b3a2 type mRNA if the *BCR* exon b3 is in the chimeric *BCR/ABL* transcript or the b2a2 type if it is not. The proteins coded differ only by the absence of 25 amino acids [1]. Several studies have examined whether the type of fusion transcript has any influence on the clinical outcome, with controversial results [2–7]. In addition, the haematological findings at diagnosis have been correlated with the two different transcripts according to a few reports [7–12]. However, the data remain controversial; in fact several groups did not succeed in demonstrating any such correlation [5,13,14]. Of note is a prospective study [7] that evidenced a correlation between the b3a2 transcript and a higher platelet count at diagnosis in a group of CML patients with a white blood cell (WBC) count at diagnosis below 100×10^9/l. In the process of cytoplasmic fragmentation of megakaryocytes and platelet formation the changes in the cytoskeleton and in cell adhesion

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and motility play an important role [15–17]. Meanwhile interactions of the *BCR/ABL* products with the cytoskeleton and molecules of the extracellular matrix are now well established. The *BCR/ABL* products interact with actin by a C-terminal domain of c-abl and the NH_2-terminus of BCR has an oligomerisation domain able to enhance the F-actin-binding activity associated with c-abl [18–21]. The chimeric protein BCR/ABL induces in the cells a redistribution of F-actin into punctate, juxtanuclear aggregates [19]. The localisation of $p210^{bcr/abl}$ on actin filaments provides the possibility of interacting directly with adhesion receptors or proteins that link these receptors to the cytoskeleton [19,21–23]. Here we report on the results obtained in 88 consecutive unselected cases of CML in chronic phase (CP).

2. Patients and methods

2.1. Study population

88 consecutive unselected patients with CML in CP were studied at presentation. Diagnostic procedures for the establishment of CML were carried out by accepted criteria. Haemoglobin level, platelet counts, leucocyte counts, blast counts were obtained using peripheral blood collected at initial examination prior to any therapy as were the bone marrow biopsy and aspirate. Cytogenetic analyses for the Ph chromosome were performed by established techniques on bone marrow cells, the same bone marrow aspirate provided mononucleated cells for the reverse transcriptase–polymerase chain reaction (RT–PCR) analysis.

2.2. Reverse transcriptase–polymerase chain reaction

Mononuclear cells were collected from the bone marrow by density gradient separation. Total cellular RNAs were extracted and then subjected to RT–PCR amplification as described elsewhere [12]. The primers used were: sense 5′GAAGAAGTGTTTCAGAAGCTTCTCCC3′ [24]; antisense, 5′TGTGATTATAGCCTAAGACCCGGAG3′ [1].

2.3. Morphometric evaluation

Bone marrow biopsies from the posterior iliac crest, routinely performed in all patients, underwent morphometric analysis in 45 out of 88 (51%) patients. Two-micron-thick sections of formalin-fixed, undecalcified, plastic-embedded bone marrow biopsies, stained with PAS to enhance morphological recognition of the megakaryocytes, were evaluated using a light microscope (Zeiss Axioskop) equipped with a videocamera (Panasonic CCDWB — BL200). Image analysis was performed using a non-proprietary software working with the frame grabber of VIDAS-PLUS (Kontron). The whole system, calibrated for each magnification with a slide marked with a micrometric line, made possible an exact equivalence between a known area and the number of pixels. Suitable fields of each biopsy were analysed at 250× magnification and the megakaryocyte size was determined by perimetrical delimitation. The frequency of megakaryocytes per square mm and their mean area were calculated for each case.

2.4. BCR/ABL structure

The amino acid sequences of the BCR/ABL proteins, b2a2 and b3a2 types, were subjected to computer predictive analysis in order to reveal possible differences in terms of secondary structure, hydrophobicity profile and domain organisation. The secondary structure prediction was performed with three different methods: PHD [25], NnPredict [26] and GOR IV [27]; the hydrophobicity profile was obtained using the Kite and Doolittle hydropathicity scale [28] and the domain organisation was analysed with SMART [29].

3. Results

3.1. Transcript type and haematological findings at diagnosis

Of the 88 Ph-positive CML patients in CP, 34 (39%) had the b2a2 type *BCR/ABL* transcript and 54 (61%) the b3a2 type. Types b2a2 and b3a2 are those that by RT–PCR give rise to amplified DNA fragments of 323 bp and 398 bp, respectively (Fig. 1). The differences reflect the lack of the 75 bp b3 exon in the former rearranged *BCR/ABL* transcript. In our series no patients expressed both b3a2 and b2a2 type transcripts. The relationship between the two transcripts and the patients' haematological findings at diagnosis was studied (Table 1) . We found that the platelet count was significantly higher ($P < 0.001$) in patients expressing the b3a2 transcript (Table 1), in agreement with other studies [7–10,12]. The other haematological parameters studied did not show any significant differences between the b2a2 and b3a2 groups. Stratifying the 88 patients on the basis of WBC below or above 100×10^9/l we still had in both groups a significant difference in the platelet count between the b2a2 and b3a2 patients (Table 1), even though in the group with WBC $> 100 \times 10^9$/l the differences in the platelet number between the b2a2 and b3a2 patients were less pronounced. A direct comparison for formal statistical analysis of all patients, stratified on the basis of WBC count, reported in [7,13,31] and this paper was not possible due to the different ways in which the data were presented. However, in the

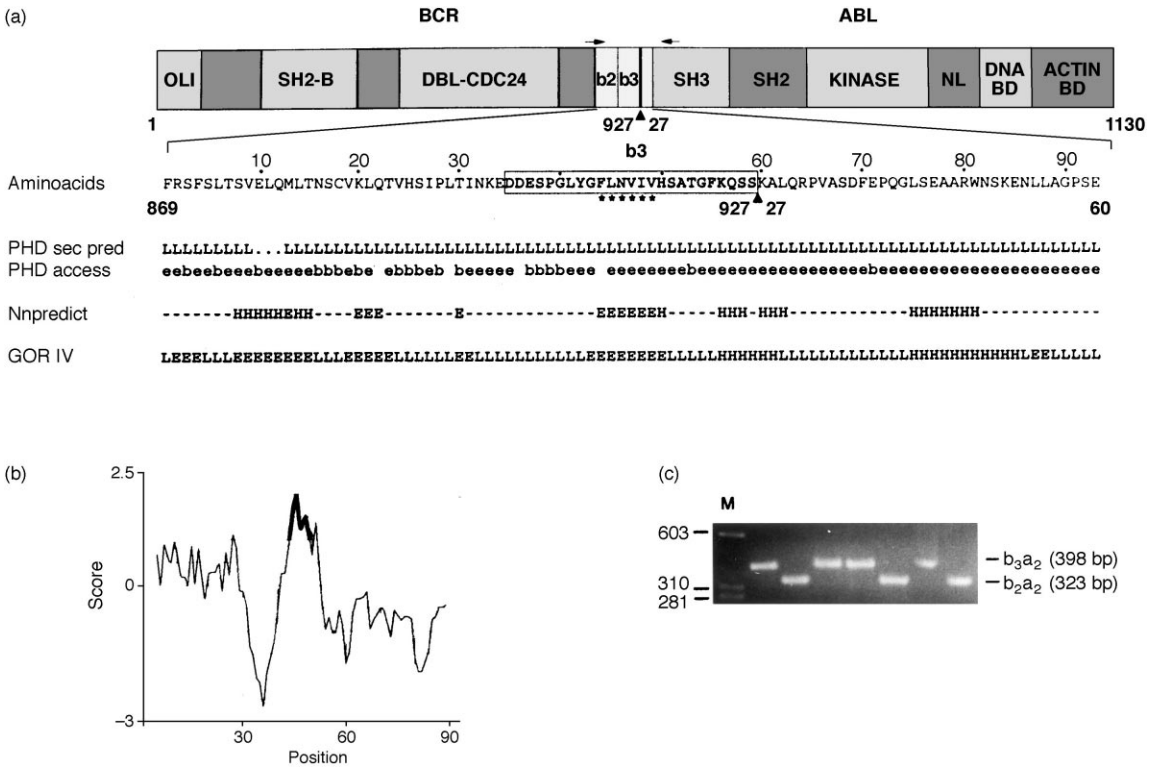

Fig. 1. (a) Schematic representation of the BCR/ABL domains. In the BCR portion of the fused protein: OLI, oligomerisation domain; SH2-B, SH2 binding region; DBL-CDC24, human dbl and budding yeast cdc24 genes homology domain; b2–b3, sequences coded by the b2 and b3 exons of the M-bcr. In the ABL portion: SH_3–SH_2, SH_3 and SH_2 domain; KINASE, tyrosine kinase domain; NL, nuclear localisation signal; DNA BD, DNA binding domain; ACTIN BD, actin binding domain. The scheme is not to scale. The arrows show the location of PCR primers. The arrowhead shows the fusion point between the BCR sequence (amino acids 1–927) [24] and the ABL sequence (amino acids 27–1130) [1]. The 93 amino acid sequence shown below represents the fusion region including the b2 exon amino acids, the b3 exon amino acids (in the box) that are missed in the protein coded by b2a2 type transcript, and part of the abl exon II amino acids. The asterisks mark the six-residue hydrophobic stretch of the b3 sequence. PHD sec pred and access [25], NnPredict [26], GOR IV [27], secondary structure prediction and solvent accessibility of the sequence shown; L, loop; e, exposed; b, buried; E, beta strand; H, alpha helix. (b) Hydrophobicity profile according to the Kyte and Doolittle hydropathicity scale [28] of the 93 amino acids shown in (a). The thick line represents the high hydrophobicity region corresponding to the amino acids labelled with asterisks in (a). (c) Representative ethidium bromide-stained 2.5% agarose gel electrophoresis of the RT-PCR amplified products of the b2a2 and b3a2 type RNA. M, ϕX174/Hae III molecular weight marker.

patients with WBC $> 100\times10^9$/l the direction of the differences in the reported platelet counts showed a similar tendency to ours (data not shown). It is to be noted that there was no marked thrombocytosis in our series: only 5 patients, all of them expressing the b3a2 type mRNA, had a platelet count at diagnosis over 1000×10^9/l. Even omitting these patients from the b3a2 group (mean $\pm$SEM: $515.1\pm33.2\times10^9$/l) the difference in the platelet count between the b2a2 and b3a2 groups remained significant ($P < 0.001$).

3.2. Transcript type and megakaryocyte size and frequency

In 45 CML patients the morphometric analysis of bone marrow biopsy showed that there was no significant difference in the frequency of megakaryocytes per square mm nor in their size (Table 2) between the b2a2 and b3a2 groups. The observed similar sizes suggest that probably the different maturational steps of megakaryocytes are equally represented in the two groups, and that also the ploidy, which correlates with cytoplasmic size, is probably the same. Moreover in the b3a2 patients with a platelet count $> 500\times10^9$/l there was no significant increase of megakaryocytes per square mm or significantly greater megakaryocyte size in comparison with the b3a2 patients with platelet counts $< 500\times10^9$/l (Table 3). Two b3a2 patients with respectively 2270 and 1900×10^9/l platelets, studied with morphometry, had lower megakaryocytes/mm^2 than the highest values found in the b3a2 and b2a2 groups, and also the mean megakaryocyte size in these two patients was smaller than the mean value of the b2a2 and b3a2 groups. In the b3a2 patients with the highest platelet counts there was a trend towards a low WBC count (Table 3) [10,13], suggesting that in these patients thrombopoietic activity may predominate over granulopoiesis mimicking the Ph-positive essential thrombocythemia (ET) [30] in which the majority of cases have the b3a2 mRNA [31].

Table 1
Characteristics at diagnosis of CML patients, in CP, expressing the b2a2 or b3a2 type of *BCR/ABL* rearranged mRNA

			Stratification of patients on the basis of WBC count			
	b2a2	b3a2	WBC < 100×10^9/l		WBC > 100×10^9/l	
			b2a2	b3a2	b2a2	b3a2
No. of cases	34 (39%)	54 (61%)	14	28	20	26
Male/female	20/14	26/28	8/6	11/17	12/8	15/11
Age (years)						
Mean±SEM	49.4±2.1	48.6±1.9				
Median (range)	51.0 (16.0–67.0)	48.0 (22.0–75.0)				
Platelets ($\times10^9$/l)						
Mean±SEM	306.1±21.1[b]	616.4±55.7[b]	334.8±31.2[c]	730.3±91.2[c]	285.9±28.3[d]	493.7±52.9[d]
Median (range)	268.5 (135.0–664.0)	523.5 (158.0–2270.0)	323.5 (195–664.0)	601.5 (194.0–2270.0)	259.5 (135.0–661.0)	397.0 (158.0–1253.0)
WBC ($\times10^9$/l)						
Mean±SEM	127.7±16.4	122.8±16.7	38.9±4.5	35.6±3.8	189.8±17.0	215.2±23.6
Median (range)	115.0 (10.5–400.0)	85.9 (5.1–700.0)	36.0 (10.5–73.8)	31.5 (5.1–87.0)	167.7 (105.0–400.0)	188.0 (119.0–700.0)
Hb (g/dl)						
Mean±SEM	12.5±0.3	12.1±0.3				
Median (range)	12.7 (7.7–16.5)	12.1 (4.0–16.2)				
Blasts[a] (%)						
Mean±SEM	4.3±0.9	4.2±0.9				
Median (range)	3.0 (0.0–19.0)	3.0 (0.0–29.0)				
Ph chromosome	34+ ve	54+ ve				

CML, chronic myeloid leukaemia; CP, chronic phase; WBC, white blood cell count; Hb, haemoglobin; Ph, Philadelphia.
[a] Peripheral myeloblasts and promyelocytes.
[b] $P<0.001$.
[c] $P=0.005$.
[d] $P=0.003$ (Student's *t*-test).

3.3. *BCR/ABL* protein type and secondary structure and hydrophobicity

The b2a2 and b3a2 type BCR/ABL protein sequences have been compared in terms of hydrophobicity, secondary structure prediction and domain composition.

Table 2
Morphometric analysis of bone marrow biopsies, and platelet count of a subgroup of patients of Table 1

	b2a2	b3a2
No. of cases	18 (40%)	27 (60%)
Male/female	11/7	16/11
Calculated Megakaryocytes/mm^2		
Mean±SEM	130.0±5.9	136.7±5.3
Median (range)	134.9 (84.5–200.2)	127.9 (106.8–191.3)
Megakaryocytes size (μm^2)		
Mean±SEM	483.1±27.7	485.7±30.2
Median (range)	469.9 (271.1–682.4)	465.8 (301.7–971.1)
Platelets ($\times10^9$/l)		
Mean±SEM	271.2±17.4[a]	612.4±92.6[a]
Median (range)	260.0 (135.0–424.0)	523.0 (158.0–2270.0)

[a] $P=0.005$ (Student's *t*-test). The microscopic fields analysed were 17.7±0.8 in the b2a2 group versus 18.3±0.5 in the b3a2 group (Mean±SEM). The area of each single field was 1.124×10^{-2} mm^2.

As all the sequences for b2a2 and b3a2 were identical this analysis refers to all 34 b2a2 patients and all 54 b3a2 patients. The different secondary structure prediction methods did not reveal any difference between the two full-length protein types whilst a discrepancy was observed in the 25 amino acids coded by the b3 exon: in one case it was seen as completely solvent accessible (e) loop region (L) (PHD method) whilst in the other cases it included a short beta strand (E) and a short alpha helix (H) (GOR and NnPredict methods) (Fig. 1a). The hydrophobicity profile (Fig. 1b), that gives information on the interaction possibility amongst protein structures, showed that the 25 amino acids corresponded to a hydrophobic region with a peak due to the six-residue sequence FLNVIV. These results were confirmed by the SMART analysis (data not shown) which found, in the b3 peptide (position 905–925 of the sequence) a possible transmembrane segment that is typically hydrophobic in proteins.

4. Discussion

The findings reported showed significant differences between the platelet counts at diagnosis (Table 1) of CML patients carrying the b3a2 or b2a2 *BCR/ABL*

Table 3
Stratification of b3a2 patients on the basis of platelet count below or above 500×10^9/l

	All b3a2 patients		b3a2 patients studied with morphometric analysis	
	Platelets $<500\times10^9$/l	Platelets $>500\times10^9$/l	Platelets $<500\times10^9$/l	Platelets $>500\times10^9$/l
No. of cases	25	29	13	14
Male/female	14/11	12/17	9/4	7/7
Platelets ($\times10^9$/l)				
Mean±SEM	322.7±18.4[a]	869.5±75.7[a]	309.5±27.6[a]	893.7±140.7[a]
Median (range)	329.0 (158.0–496.0)	779.0 (508.0–2270.0)	288.0 (158.0–477.0)	712.5 (523.0–2270.0)
WBC ($\times10^9$/l)				
Mean±SEM	146.5±28.3[b]	102.5±19.0[b]	174.3±49.4[c]	74.1±17.4[c]
Median (range)	131.0 (15.4–700.0)	49.1 (5.1–405.0)	140.0 (33.6±700.0)	49.8 (5.1±210.0)
Calculated Megakaryocytes/mm^2				
Mean±SEM			127.2±5.2[b]	145.6±8.6[b]
Median (range)			124.6 (106.8–164.6)	142.4 (106.8–191.3)
Megakaryocytes size (μm^2)				
Mean±SEM			448.2±23.9[b]	520.6±53.3[b]
Median (range)			442.6 (303.3–580.1)	469.7 (301.7–971.1)

[a] $P<0.001$ (Student's t-test).
[b] Differences non significant.
[c] $P=0.06$.

transcripts, the b3a2 transcripts being associated with higher values. The debate on the relationship between *BCR/ABL* transcript type in CML and platelet count at presentation has now been going on for several years with contrasting results [5,7–14], and the discordance has been thought to be due to qualitative or quantitative differences in patient or sample selection. To understand this controversial, but documented, clinical finding it would be important to dissect the molecular and cellular events involved in the biological process of platelet formation, to see if it is possible to hypothesise interactions between *BCR/ABL* functions and thrombopoiesis. The proposed models of platelet formation consist of several steps including: the formation of the Demarcation Membrane System (DMS) by invagination of the megakaryocyte membrane and the adherence of cells to the extracellular matrix; the formation of pseudopodia with rearrangement of the actin-rich peripheral zone, which permits microtubule elongation, orientation and bundling; the movement of the DMS and cytoplasmic granules and fragmentation of proplatelets and platelets [15–17]. It is thus evident that thrombopoiesis is closely related to the interaction of the actin and microtubule systems with megakaryocyte integrins and the extracellular matrix. Findings obtained by treating guinea-pig bone marrow megakaryocytes with cytochalasin D [15], which shortens actin filaments, showed an acceleration of platelet formation probably because cytochalasin D by disrupting the actin-rich peripheral zone allows microtubules to expand in the previously actin-dense areas. The way in which the BCR/ABL protein structure might influence the process of platelet formation is probably related to the well-proven interactions of BCR/ABL protein with cytoskeleton, integrin and extracellular matrix molecules [18–23]. A tetramerisation domain, present in the NH2-termini of the BCR protein sequence [21], promotes clustering of a C-terminal actin-binding domain of the c-abl sequence [19] (Fig. 1) that cross-links actin filaments [21]. BCR/ABL can induce a redistribution of F-actin from the cortical cytoskeleton into punctate, juxtanuclear aggregates [19]. The oligomerisation also plays a role in the activation of the BCR/ABL tyrosine kinase [21] with a BCR domain that is able to bind to the c-abl SH2 domain, forcing it, by protein folding, into a more active and accessible conformation [21,32] (Fig. 1). Although it is not possible to draw any conclusions about changes induced by the lack of b3 exon amino acids, with the six-residue hydrophobic stretch, one can hypothesise that the absence of this region might be responsible for some spatial rearrangements of the adjacent domains or, owing to the absence of the hydrophobic characteristics of this sequence, for altered cellular interactions. The multiple stimuli involved in platelet production may include BCR/ABL protein types with different conformations as a result of a lack of the b3 exon amino acids, and consequently different activity [32]. These facts might determine a different efficiency in actin microfilament redistribution from the actin-rich peripheral zone, influencing microtubule elongation and then the time course of platelet formation. In our series the difference in platelet counts is not related to the number of megakaryocytes or to their sizes, even in the patients with the highest platelet count (Table 2). These findings let us hypothesise, although we do not have information on the platelet half-life, that these differences might be

due to a different efficiency in platelet production. It is to be noted that in the literature [10,30], in our series and in the rare patients with Ph positive ET [31] the highest platelet counts correlate with the b3a2 type transcript. It is, however, worthy of note that in the b3a2 patients there is a subgroup with platelet counts not different from those of the b2a2 group (Table 3). In the multifactorial process of platelet production many signalling pathways, that interact with each other at different levels, should be involved and the type of BCR/ABL rearranged product is not sufficient alone to explain the increment of thrombopoiesis. Probably the signal transduction role of BCR/ABL protein, where present, would be only a variable that in certain cases could influence the phenomenon. An example of this multiple involvement of different pathways in thrombopoiesis is provided by ET, a clonal disorder characterised by thrombocytosis and abnormal proliferation of megakaryocytes and in which the BCR/ABL protein, in the greatest number of cases, is not present. In this disease it seems that an altered expression of thrombopoietin (TPO) and its receptor c-Mpl or an altered sensitivity of the TPO-c-Mpl system may play a role [33] although their exact involvement in thrombopoiesis is still elusive. Among the variables that could influence the process we are now trying to investigate possible correlations between the platelet count at diagnosis with the amount of rearranged transcript and with the $p210^{bcr/abl}$ protein level. In conclusion, our findings confirming that the b3a2 type transcript is associated with a significant excess of thrombopoiesis in a subpopulation of p210 CML patients are not fortuitous and suggest that the relationship between BCR/ABL protein structure and thrombopoiesis merits further study.

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