PERSISTENCE OF PH+/CD34+ CELLS IN CHRONIC MYELOID LEUKEMIA PATIENTS IN PROLONGED COMPLETE CYTOGENETIC REMISSION FOLLOWING IMATINIB MESYLATE TREATMENT

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INTRODUCTION

Chronic myelogenous leukemia (CML) is characterized by a clonal expansion of a hematopoietic stem cell possessing a reciprocal translocation between chromosomes 9 and 22, the Philadelphia chromosome, as identified cytogenetically or molecularly (RT-PCR). CML accounts for 15% of adult leukemias. The disease progresses from a chronic phase through an accelerated phase to a blast phase. In the past, the National Comprehensive Cancer Network had suggested that there were three primary treatments available for CML which included: allogeneic bone marrow transplantation (BMT), IFN-α with or without cytarabine, and imatinib mesylate (Glivec®). It has been shown that Glivec is superior to the combination of interferon plus cytarabine (1,2). Although BMT can be a curative treatment for CML it is not usually used as a front-line therapy, due to limited donor availability and high toxicity of the procedure. Five-year survival rates following HLA-matched transplants are approximately 75% for patients in chronic phase.

Glivec, the first developed inhibitor of the BCR-ABL tyrosine kinase, was approved in 2001 by the Food and Drug Administration for the treatment of CML in chronic phase and it has proven highly effective (3-5). In fact, studies have shown that imatinib mesylate can induce hematologic responses in excess of 90% and estimated complete cytogenetic responses in more than 80% at a median follow-up of 19 months.

Once Complete Cytogenetic Response (CCR) has been obtained (median time 5.5 months after imatinib start), RT-PCR is crucial in determining an accurate response to treatment, monitoring minimal residual disease, and detecting relapse. RT-PCR is a highly sensitive assay, which has the ability to detect one leukemia cell in the background of 10^5–10^6 normal cells. By RT-PCR the majority of imatinib treated patients still show detectable disease thus suggesting that imatinib mesylate usually cannot completely cure this disease (6,7). The most reasonable explanation is that Ph+ stem cells are insensitive to imatinib (8,9). As a clinical consequence, even in those patients in which BCR-ABL transcript is undetectable by the most sensitive nested RT-PCR method (complete molecular response), discontinuation of the drug is followed by disease recurrence in nearly all cases (10,11). On this regard Bathia et al. (12), have shown that exposure to imatinib may not completely eliminate leukemic progenitors and that BCR-ABL–positive stem cells can be detected in patients in CCR after short term of imatinib treatment. The most common marker for myeloid stem cell is CD34 surface antigen and thus co-expression of CD34 and BCR-ABL is a proper combination to identify CML precursors in the bone marrow. Through this analysis, Bathia an colleagues found in 12/15 patients studied after a median time of 10 months of imatinib a median of 11% of residual CML CD34+ progenitors in the bone marrow while only 3/15 had no measurable residual CD34+ cells (12). Quiescent primitive CML progenitors may be resistant to apoptosis following imatinib exposure (13) or they may be resistant to the drug through mechanisms such as increased drug efflux activity (14) and altered transporter genes expression (15). Furthermore detection of BCR-ABL kinase mutations in CD34+/Ph+ cells have been detected (16). While the mechanism underlying Ph+ progenitors resistance to imatinib has been extensively studies in vitro model, no data are currently available regarding the real incidence of leukemic precursors persisting in patients after prolonged treatment with this drug. Thus, we evaluated the amount of bone marrow residual CD34+/Ph+ cells in 31 CML patients in stable long lasting CCR during imatinib treatment.

MATERIAL AND METHODS

CML patients with sustained CCR and at least 24 months of continuous imatinib treatment were evaluated for residual CD34+/Ph+ cells. 20ml of bone marrow samples were obtained from each patient after receiving informed consent. Part of the sample was not manipulated and was evaluated for conventional cytogenetics, FISH analysis, molecular biology studies and flow cytometry study. The rest of the sample was used for CD34+ separation and subsequent FISH analysis of CD34+ purified cells. Bone marrow mononuclear cells (BMMCs) were isolated by density gradient separation and CD34+ cells were selected from BMMCs using immunomagnetic column separation according to published methods and manufacturer instructions (Miltenyi Biotech, Auburn, CA) (17). Flow cytometry analysis to confirm CD34+ cells purity after separation was performed by incubating each cell sample with an anti CD34 fluorescent antibody and subsequently by analyzing the samples on a FACScan flow cytometer (BD Biosciences, San Josè CA USA). FISH analysis of whole bone marrow cells as well of sorted CD34+ cells was performed on fixed cells according to conventional published methods and manufacturer's specifications. LSI BCR/ABL Dual color extra signal (ES), single fusion translocation was used as probe (Vysis, Downers Grove,IL, USA). Slides were analyzed with a Nikon 2 fluorescence microscope and images captured with a

CCD camera using image analysis system (Genikon). When conventional bone marrow FISH analysis was performed, at least 300 interphase cells were analyzed independently by two different observers, while for purified CD34+ cells a minimum of 100 interphase nuclei per each sample were evaluated. Each bone marrow sample has been aslso evaluated for molecular biology studies through a standardized Taqman based Q-RT-PCR technique for BCR-ABL transcripts. Results were expressed as BCR-ABL/ABL ratio. In case of undetectable level of BCR-ABL by Q-RT-PCR a nested qualitative RT-PCR was also evaluated (18).

RESULTS AND DISCUSSION

A total of 31 CML patients were evaluated in our study. The median age was 46 (range 19-77) with 18 male and 13 female. At imatinib start all patients were in Chronic Phase (CP), 15/31 were newly diagnosed while 16/31 have been previously refractory (11 patients) or intolerant (6 patients) to Interferon-alpha. At the time of residual CD34+/Ph+ evaluation all patients were on 400mg/day of imatinib for a median time of 39 months (range 24-59). All of them had a previously documented and sustained CCR for a median time of 35 months (range 15-53) that was again confirmed by conventional bone marrow cytogenetic analysis of at least 20 metaphases in all patients. Whole bone marrow FISH analysis identified Ph+ cells in 11/31 (35%) (median of 2% nuclei, range 1-4%) while BCR-ABL transcript was still measurable in 24/31 (77%) patients by RT-PCR. After immunomagnetic sorting of about 10 ml of marrow aspirate an adequate number of CD34+ cells (average $9.6x10^5$) for FISH analysis was collected in all 31 patients and CD34+ enriched population was confirmed to be more than 90% pure by flow cytometry analysis. FISH analysis of CD34+ purified cells showed the persistence of Ph+ cells in 14/31 (45%) patients with a median number of 1% (range 1-7%). Fig.1 shows a FISH image of a leukemic CD34+ cell (BCR-ABL positive) and a normal CD34+ bone marrow cell.

Fig.1 BCR/ABL CD34+ (A) and normal CD34+ (B) cells detected by FISH with LSI BCR/ABL dual color extra signal single fusion translocation probe.

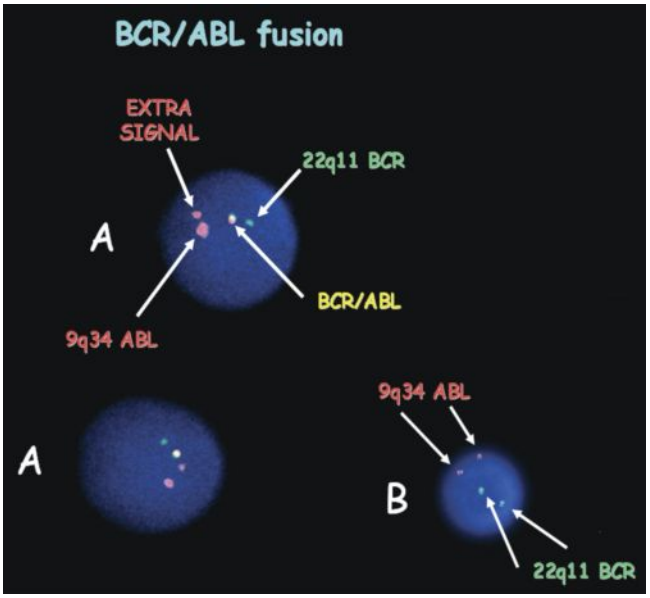

Table 1 shows disease characteristics and follow-up of 14 CD34+/Ph+ positive patients and of 17 CD34+/Ph+ negative patients.

At the time of evaluation a weak correlation was found between the persistence of leukemic stem cells and the amount of molecular residual disease. In fact bone marrow fusion transcript was still detectable in 12/14 (86%) CD34+/Ph+ positive patients and in 12/17 (70%) CD34+/Ph+ negative patients. All patients continued imatinib at 400mg/day and were monitored for their residual disease. Interestingly, after a median follow-up time of 20 months (range 7-36) since CD34+ evaluation, 4/14 (28%) patients with residual CD34+/Ph+ positive cells showed a significant increase of their molecular disease (considered as more than 1 log increase of BCR-ABL/ABL ratio in two consecutive samples) (Table 1. pt # 1,3,8,9). In addition, one of them lost CCR while 4/14 (28%) were in stable complete molecular response (CMolR). On the contrary, only 2/17(11%) patients without residual Ph+ stem cells showed an increased level of detectable molecular disease during follow up (Table 1. pt #20, 28). No patient in this group lost CCR and 9/17(53%) were in CMolR. To our knowledge this is the first "in vivo" evaluation confirming that CML precursors survive during imatinib treatment even after prolonged and stable CCR. Our findings assessed that at least 45% of these patients harbor residual CD34+/Ph+ cells although the role of these cells in the outcome of imatinib treated CML patients is not clearly definable from our study. Albeit the series of patients is small we found a trend in a rise of molecular residual disease over time in patients with CD34+/Ph+ cells, with potentially a higher risk of cytogenetic relapse. On the other hand at least 4 patients with documented residual CML precursors achieved a stable CMolR. The coexistence of bone marrow CML precursors and undetectable transcript may reinforce a recent hypothesis indicating a very low level of expression of BCR-ABL of quiescent CML cells (19).

In further studies, the evidence that CML stem cells may be documented by FISH, even in the absence of detectable level of BCR-ABL, may identify a new role for molecular cytogenetics in the quantification but also characterization of the cell compartment resistant to imatinib, potential source of disease relapse.

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Table 1. Disease's characteristics at time of evaluation and follow-up of patients with CD34+/Ph+ residual cells (from pt#1 to #14) and patients without CD34+/Ph+ residual cells (from pt#15 to #31).

	Sex	Age	Pre-treated (IFN-α)	Months on imatinib	Months of CCR*	RT-Q-PCR°	FISH BM # (% Ph+)	FISH CD34+/Ph+§ (%)	Follow-up CCR (**)	Follow-up RT-Q-PCR°
1	M	70	Yes	39	36	1.2	4	7	Lost CCR +51	5.7
2	M	47	Yes	25	21	0.3	1	2	CCR +42	0.6
3	M	62	Yes	39	33	0.07	0	2	CCR +61	1.4
4	F	32	Yes	31	15	0.6	4	1	CCR +48	0.8
5	F	65	No	26	21	0.035	2	1	CCR +45	Nested neg
6	F	49	Yes	48	45	0.05	2	1	CCR +67	0.001
7	F	77	No	38	35	Nested neg°°	0	2	CCR +57	Nested neg
8	F	65	No	37	34	0.01	0	1	CCR +47	0.4
9	F	34	Yes	55	26	0.05	0	1	CCR +58	0.9
10	M	43	No	40	37	0.15	0	1	CCR+51	0.09
11	F	45	No	56	51	Nested neg	1	1	CCR +78	Nested neg
12	F	67	Yes	50	47	0.07	1	4	CCR +64	0.02
13	M	65	No	57	51	0.06	3	5	CCR +71	0.01
14	F	28	No	24	21	0.07	0	1	CCR +38	Nested neg
15	M	49	no	24	21	0.008	0	0	CCR +41	Nested neg
16	M	44	NO	30	27	0.03	0	0	CCR +39	0.04
17	F	60	SI	29	26	Nested neg	0	0	CCR +51	Nested neg
18	M	63	SI	37	32	Nested neg	0	0	CCR +51	Nested neg
19	F	57	NO	49	28	Nested neg	0	0	CCR + 64	Nested neg
20	M	45	SI	58	52	0.03	0	0	CCR +77	0.1
21	M	69	NO	25	22	Nested neg	0	0	CCR +29	Nested neg
22	M	31	NO	51	44	0.005	0	0	CCR +63	0.003
23	F	49	SI	49	43	0.01	0	0	CCR +60	0.01
24	M	40	SI	36	33	1.2	1	0	CCR +46	0.6
25	M	34	SI	36	33	0.005	4	0	CCR +55	0.0035
26	M	32	SI	59	51	0.2	0	0	CCR+62	Nested neg
27	M	28	NO	29	26	0.2	0	0	CCR +33	0.09
28	M	68	SI	56	53	0.03	4	0	CCR +75	1.32
29	M	19	SI	49	44	Nested neg	0	0	CCR +52	Nested neg
30	M	40	NO	24	15	0.001	0	0	CCR +35	Nested neg
31	F	40	SI	57	51	0.03	1	0	CCR +71	Nested neg

Legend:
**CCR complete cytogenetic response; **Month on imatinib; °Whole bone marrow Quantitative RT-PCR analysis measured as BCR-ABL/ABL ratio by standardized Taqman based Q-RT-PCR technique; °°Absence of BCR-ABL transcript in the bone marrow even after qualitative nested RT-PCR; #Whole bone marrow FISH analysis.*

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