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Southern Technique and Cytogenetics Are Complementary and Must Be Used Together in the Evaluation of Ph1, M-BCR Positive Chronic Myeloid Leukemia (CML) Patients Treated With Alpha Interferon (IFN-ALPHA)

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Cytogenetic analysis is the gold standard for the follow-up of CML patients. The sensitivity of cytogenetics is fairly similar to that of Southern detection of M-BCR rearrangement (5%); this last technique has the potential advantage of being independent of cell division and yield of metaphases. IFN alpha treatment can induce lack of growth of hemopoietic precursors and poor yield of metaphases has been observed. For this reason we decided to study the grade of concordance and complementarity between analysis of karyotype and detection of M-BCR rearrangement of Southern blot. We studied 43 Ph1 positive, M-BCR positive pre-BMT CML patients (48 samples) treated with IFN alpha 2a. Karyotype was done on bone marrow cells by direct method, culture, and banding. Southern technique was performed onto DNA from peripheral blood leukocytes treated with BglII (and XbaI if necessary) and hybridized with the universal probe (Ph1/bcr-3, Transprobe 1) labelled with dCTP32.

A highly significant association between both tests was obtained. Of 48 samples analyzed, 34 were evaluable by both methods and 28 gave the same result for both tests. The concordance between the tests was good (kappa index: 0.63). Of total samples 27.1% was not evaluable by cytogenetics; this figure was 31.2% in samples from patients who were previously in complete cytogenetic response. All of the specimens not evaluable by karyotyping were evaluable by Southern. One sample was not analyzable by Southern but it was evaluable by cytogenetic analysis. The information obtained by Southern technique was clinically relevant, and decisions were made according to its results.

We conclude that both tests show a significant association and a good concordance, although they are not interchangeable. Cytogenetic and molecular studies are complementary and must be employed together in CML patients treated with alpha-interferon.

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Key words: chronic myelogenous leukemia, interferon alpha, cytogenetics, M-BCR, Southern

INTRODUCTION

Chronic myeloid leukemia is a clonal myeloproliferative disorder of the primitive haematopoietic stem cell [1]. It displays a cytogenetic hallmark in more than 95% of cases: the Ph1 chromosome that results from the reciprocal translocation t9:22(q34;q11) which at a molecular level represents the bcr-abl rearrangement [2]. With IFN alpha treatment 70% of haematologic responses and 15–20% of major (complete and partial) cytogenetic responses can be obtained [3]. Obtaining a sustained major genetic response in IFN-treated CML patients is crucial

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because of its prognostic value. A longer survival for patients with IFN-induced major genetic response has been suggested [4] and patients with complete genetic response have an actuarial probability of survival without genetic relapse in excess of 90% [5]. Certifying a complete genetic response is thus the major issue in the followup of CML patients treated with alpha-IFN.

Karyotype and molecular techniques (PCR and Southern blot) can be employed to determine the grade of leukemic Ph1 clone suppression in IFN treated CML patients [6,7]. Although karyotype is the gold standard for monitoring IFN treated CML patients [3] this technique requires a bone marrow sample; besides it needs the growth of an adequate number of metaphases [8]. Southern detection of M-BCR rearrangement has a sensitivity similar to karyotyping (5–10%) [6] and is a laborious and long technique but it has the advantage of not requiring bone marrow (it can be performed on peripheral blood leukocytes) and of being independent of cell division [6]. This last point is of importance because IFN treated patients may show lack of growth of metaphases in karyotyping [9]. After having detected this problem in some of our first patients, our group decided to study samples from IFN alpha-treated CML patients in order to appraise the concordance and complementarity between cytogenetic analysis and detection of the M-BCR region rearrangement by Southern blot.

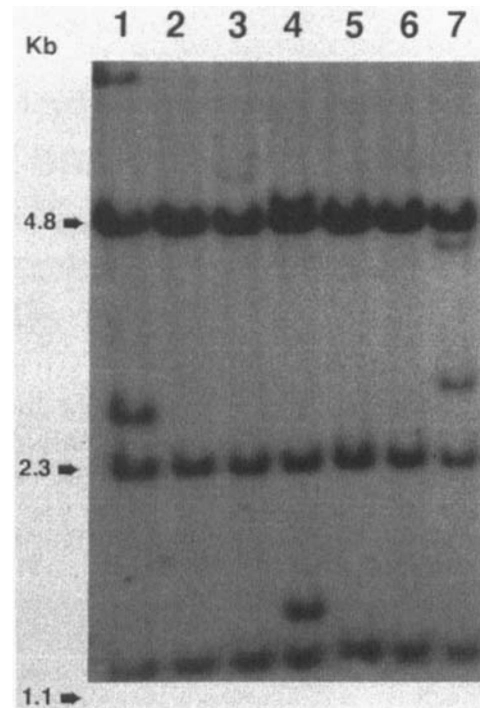

Fig. 1. Southern blot analysis. Autoradiographs of Southern blot hybridized with the phl/bcr-3 probe. Lanes 2,5,6, show normal BglII fragments. Lanes 1,3,4,7 represent normal and rearranged DNA fragments.

PATIENTS AND METHODS

Patients

We have studied 43 patients diagnosed of chronic myeloid leukemia Ph1, M-BCR positive in first chronic phase. All of them were treated with interferon alpha 2a (Roferon-A): 5.4 ± 3 million U/d for a median time of 711 days (range: 24–1,826). The number of samples analyzed as 48, of which 34 were from patients in complete hematologic remission and 16 were from patients in previous complete cytogenetic remission. We did not discontinue IFN treatment before obtaining bone marrow or blood specimens. Informed consent was obtained according to local-ethic committee guidelines.

Methods

The karyotype analysis was carried out on bone marrow cells extracted in heparin. Some cells were cultured in RPMI1640 medium supplemented with 18% fetal bovine serum for 2 hr; then colchicine was added followed by KCL 0.075 M and afterwards they were fixed with Carnoy and observed (direct method). The rest of the cells were cultured for 24 hr and G banding technique was accomplished [10].

Cytogenetic response to IFN was classified according to Talpaz et al. [3]: CGR was defined as no Ph positive metaphases; partial response (PGR) indicated 5–34% Ph positive metaphases; a minimal cytogenetic response was defined as presence of the Ph chromosome between 35 and 95% of metaphases.

Southern blot was performed onto DNA extracted from peripheral blood leukocytes by standard method (phenol-chlorophorm extraction and precipitation with etanol) [11]. DNA was digested with the restriction enzymes BglII and Xbal if necessary (in less than 1% of cases abnormal restriction fragments may not be visible after digestion with BglII), electrophoresed in 0.8% agarose gel, transferred to a nylon membrane (Gene Screen Plus, Du Pont, Wilmington, DE) in 0.4 N NaOH and hybridized with the universal probe (Ph1/bcr-3 Transprobe 1, Oncogene Science, Manhasset, NY) labelled with dCTP32 [12]. The technique was qualitative and densitometry of bands was not performed. An autorradiography sample is shown in Figure 1.

Statistical methods. For statistical consideration, a cytogenetic analysis was scored as non-evaluable when no metaphases were obtained, as positive when Ph1 positive metaphases were observed, and negative when no Ph1 metaphases could be visualized; the target number of metaphases to be counted was 20. Southern results were classified as non-evaluable when no bands were present or the intensity of them was too faint to conclude, as positive in the presence of additional bands, and as

TABLE I. Previous Cytogenetic Response to IFN Classified According to Talpaz et al. [3]

Cytogenetic response (CR)	N
Complete	16
Partial	2
Minor	11
No CR	17
Not assessed	2

TABLE II. Number of Metaphases Obtained From Bone Marrow Aspiration

No. of metaphases	N
Zero	13
1–10	1
11–20	12
>20	15
Not stated	7

TABLE III. Association and Concordance of Southern and Karyotype*

	Karyotype	
	Phl positive	Phl negative
Southern		
Positive	17	2
Negative	4	11

*Chi square: 13.59 $P < 0.0002$; kappa index: 0.66.

TABLE IV. Evaluable Results by Each Technique*

	Karyotype	
	Evaluable	Not evaluable
Southern		
Evaluable	**34**/*10*	**13**/*5*
Not evaluable	**1**/*1*	**0**/*0*

*Total samples (n = 48, bold type) and samples from patients with previous CGR (n = 16, italics).

negative when only the germ line bands were observed. The association was determined by the Chi2 test and the concordance was estimated by the kappa index.

RESULTS

We analyzed 48 samples from 43 patients of chronic phase CML treated with alpha 2a IFN (mean + SD: 5.4 ± 3 million U/d) for a median time of 711 days (range: 24–1,826). Out of 48 specimens, 34 were from patients in complete hematologic remission, 9 from patients with partial hematologic response, and 5 belonged to individuals with no response. Table I shows the previous cytogenetic response the patients obtained before the problem sample was obtained. It can be seen that 16 out of 48 samples were from patients who have had a previous CGR. The number of metaphases obtained is depicted in Table II; it is important to point out that no metaphases could be obtained in 13 cases. As a control, it must be pointed out that in 15% of 120 samples from patients with several hematologic diseases not receiving IFN, the growth of metaphases could not be achieved.

Concordance and Association

Of 48 samples analyzed, 34 were evaluable by both methods and 28 gave the same result for both tests. We found a highly significant association between cytogenetic analysis and Southern by means of the Chi 2 test (Chi2 = 13.59; $P < 0.0002$) (Table III). The kappa index was 0.63; this value shows a fairly good concordance between the tests.

Complementarity

Of total samples 27.1% was not evaluable by karyotype; out of 16 samples from patients with previous CCR, 5 were not analyzable by karyotype. All of them could be evaluated by Southern. One sample that could not be evaluated by Southern was evaluable by karyotyping (Table IV).

Clinical Relevance

The clinical value of the detection of M-BCR rearrangement in the setting of non-evaluable cytogenetics is depicted in Table V, where the results of the Southern technique are compared with the immediately previous and next cytogenetic response.

Patient 22 was treated with alpha IFN after relapsing post BMT. MBCR was rearranged at this moment (Fig. 1, lane 4). In this patient absence of MBCR rearrangement allows us to certify the complete response, sparing the need of alternative treatment and of marrow punctures (Fig. 1, lane 5). This patient continues in IFN maintained CGR, 84 months after IFN was started and 69 months after obtaining the CGR. Her evolution has been previously described [13]. No bcr-abl transcript is detected by double step-RT-PCR (data not shown) [14].

Cytogenetic studies on patient 16 were extremely disappointing due to frequent lack of metaphases, and we used Southern technique in order to monitor her complete response, which persists more than 70 months after its detection.

Patient 72 is a child who obtained a complete cytoge-

TABLE V. Results Obtained by Southern (Central Column) in Samples Which Were Coincidental With a Non-Evaluable Cytogenetic Exam (i.e., No Metaphases Were Obtained)*

Patient no.	Previous GR [% Phl (no. met)]	MBCR	Next GR [% Phl (no. met)]
6	90 (19)	Rearranged	100 (NS)
5	66 (20)	Rearranged	45 (20)
8	5 (18)	Rearranged	10 (20)
15	Not evaluable	Rearranged	85 (NS)
16	0 (40)[a]	Not rearranged	Not evaluable
16	Not evaluable	Not rearranged	0 (25)
16	0 (25)	Not rearranged	0 (20)
20	100 (25)	Rearranged	Not evaluated
22	0 (15)	Not rearranged	0 (15)
35	100 (13)	Rearranged	Not evaluated[b]
44	100 (NS)	Rearranged	100 (22)
72	15 (27)	Not rearranged	Not evaluated[b]
10	100 (12)	Rearranged	100 (NS)

*The left column shows the previous result obtained by cytogenetics in the same patient, and the right column shows the cytogenetic results of the next immediate exam, if available. NS, not stated.
[a]In this patient, three exams were not evaluable by cytogenetics at dates 12/91, 10/92, 11/93.
[b]These patients were submitted to BMT.

netic response after IFN. IFN was stopped in order to collect bone marrow, but Phl metaphases increased to 15% during this period. IFN was restarted after the bone marrow harvest and a disappearance of MBCR rearrangement was reinduced; at that moment an unrelated bone marrow donor was found. BMT was performed while on CGR, and the patient is currently alive 18 months after BMT.

Patients 20 and 35 were patients with poor tolerance to IFN. Absence of complete response in MBCR rearrangement contributed to the discontinuation of the drug on these patients.

DISCUSSION

Cytogenetic studies are the gold standard for monitoring the treatment of Phl positive CML patients. Southern detection of MBCR rearrangement in peripheral blood leukocytes and bone marrow karyotype have been compared in patients with CML in chronic phase treated with chemotherapy. In these studies, the results of MBCR rearrangement analysis in peripheral blood leukocytes showed a good concordance with those of bone marrow karyotype (kappa index = 0.65) [15].

Detection of disappearance of Phl chromosome or its molecular counterpart, the MBCR rearrangement, has an important clinical impact in CML patients treated with IFN alpha, because complete cytogenetic responses seem to be associated with longer duration of hematologic responses [3] and better survival [4]. Moreover, patients in complete cytogenetic remission could be eligible for marrow collection and, eventually, for autologous transplantation [16,17].

Qualitative PCR seems of limited utility in these patients because most patients with a complete cytogenetic response show presence of the abnormal transcript bcr-abl [18]. Preliminary reports recently claim that quantitative PCR seems to have definite advantages over karyotyping, although difficulties in its standardization may hamper its widespread use [19]. Interphase-fluorescent in situ hybridization (iFISH) has the theoretical advantage of not needing the yield of cell divisions; however, recent reports comparing metaphase FISH and interphase FISH in CML patients have shown that iFISH overestimates the degree of cytogenetic response [20].

Therefore, karyotyping and detection of MBCR rearrangement are the mainstays of the follow-up of CML patients. Contrary to quantitative PCR and FISH, these techniques are available to most medium-sized hematology departments. To our knowledge, our study is the first which is addressed to assess the concordance of these two techniques in IFN treated CML patients. Our results seem to indicate that interferon alpha treatment in CML patients is frequently associated with a poor yield of metaphases; this fact could reflect the delaying effect of IFN on the cell cycle [21]; alternatively, it may result from the myelosuppression induced by IFN. In our series, 50% of samples had a poor marrow cellularity and in half of them no metaphases were obtained.

Although no change in overall bone marrow cellularity was found in patients with solid tumors treated with IFN alpha for a short period of time [22], several authors have observed that "emptiness" of bone marrow samples is a frequent finding in IFN-treated CML patients [23]. Myelosuppression is a rather frequent secondary effect of IFN alpha in CML and even aplasia has been described in IFN alpha treated Phl positive CML patients that had previously received alkylating agent [24]. A poor yield

of metaphases could lower the predictive power of the technique [8]; the absence of growth of metaphases may even indicate a new bone marrow aspiration.

In our study we found that Southern and karyotype analysis show a very significant association. The kappa index greater than 0.6 demonstrates that the concordance between the tests is good. However, Southern detection of MBCR rearrangement cannot replace cytogenetics in monitoring these patients. Besides, karyotyping permits the demonstration of other chromosomic anomalies that may be of prognostic significance [25], and gives a quantitative estimate of IFN effect; this makes an advantage over the qualitative Southern technique we have employed. Quantitative analysis of MBCR rearrangement has been thoroughly standardized by Ayscue et al. and Grossman et al. who applied this technique to monitor the IFN alpha treatment in three and six patients, respectively [26,27]. Few samples non-evaluable by Southern are expected (for instance, only one out of 48, in our series), and we can anticipate that quantitative Southern may be a most valuable tool in the monitoring of these patients. It is conceivable that karyotype and MBCR analysis may reflect different biological phenomena in CML patients treated with IFN alpha, having potentially different populations as targets; dividing cells and total cells, respectively. Further studies are necessary to clarify this point, and its clinical relevance. Recently, Verschraegen et al. have applied a quantitative Southern technique in a very large group of patients, and their results showed an excellent correlation between the two techniques. This study included only samples evaluable by cytogenetics (i.e., it did not include samples without yield of metaphases) [28].

Conversely, our study was addressed to ascertain the degree of complementarity, that is, if Southern would allow to evaluate samples which were not evaluable by cytogenetics, as a result of poor yield of metaphases. We have found that 27.1% of total samples were not evaluable by cytogenetics, but they were successfully analyzed by Southern. This technique has been very useful and clinically relevant in patients with previous complete genetic responses; in these patients karyotype was not evaluable in 5 out of 16 samples, whereas Southern was informative in all of them. This information allowed us to take clinically important decisions, such as delaying donor lymphocyte infusions (IDN 22) or continuing with IFN (IDN 16) (see Results).

We conclude that Southern and cytogenetics are complementary and must be used together in the follow-up of IFN alpha treated chronic phase CML patients. This seems to be especially true in patients with previous CGR; in these patients a poor yield of metaphases is rather common, and, if the result of MBCR rearrangement is negative, new and disturbing bone marrow sampling could be spared, and clinical decisions could be taken on its basis.

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