Polymerase chain reaction based assay to detect allelic loss in human DNA: loss of β -interferon gene in chronic myelogenous leukemia

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Received September 22, 1989; Revised and Accepted January 4, 1990

ABSTRACT

We have developed a polymerase chain reaction (PCR) based technique to detect allelic loss. In this differential PCR a target gene and a reference gene are coamplified in the same reaction vessel. The ratio of the intensity of the two resultant bands is an indication of relative gene dosage. This procedure is sensitive in that gene copy ratios of 2:1 and 3:2 (reference: target gene) can readily be detected. Using this differential PCR, we have examined 64 cases of chronic myelogenous leukemia (CML) for the loss of the β 1-interferon gene, a relatively common event in certain human leukemias and lymphomas (1). Only one patient who was Philadelphia chromosome positive and who was in blast crisis exhibited allelic loss of the β -interferon gene. Thus despite deletions at the β -interferon locus in the CML cell line, K562, this perturbation is rarely seen in primary CML samples.

INTRODUCTION

The loss of tumor-suppressor genes has been implicated in carcinogenesis (2). Cells from human acute lymphocytic leukemia (ALL), non-Hodgkin's lymphomas, and their derivative cell lines frequently exhibit a loss of a band in the short arm of chromosome 9 (9p22) (3). The genes encoding the human α and β 1-interferon genes have been mapped to this area (9p22) (4) and the homozygous deletion of these genes in human leukemia cell lines as well as in 9% of primary ALL samples has recently been documented (1). Besides lymphoblastic cell lines, the CML-derived cell line, K562, also carries the homozygous loss of the α and β -interferon genes (1) suggesting that deletions at this locus may be important in the conversion from chronic phase to blast crisis. To test this hypothesis, we sought to detect allelic loss of the β -interferon gene in a large number of CML samples both in chronic phase and in blast crisis. Though Southern blot hybridization (5) is an appropriate method for detecting homozygous deletions, we found this technique less sensitive in detecting subtle changes in gene copy number for reasons

previously enumerated (6). Recently, we have developed a polymerase chain reaction (PCR) based method called differential PCR to quantify gene amplification (7). In the present report, we describe an adaptation of differential PCR for the detection of allelic loss at the β -interferon locus. Applying this procedure to CML samples, we detected hemizygosity of the β -interferon gene in only one of 64 patients. Thus deletions at this genetic locus is not frequently associated with progression to blast crisis.

MATERIALS AND METHODS

Cells: The cell line K562 was obtained from the American Type Tissue Collection (ATCC). Patient cells: Diagnosis of CML was made using standard blood and bone marrow smears. The presence of Philadelphia chromosome or bcr/abl rearrangement was required for inclusion into this study. We investigated DNA from 64 patients; 49 patients were in chronic phase, and 15 patients were in blast crisis. DNA was extracted from blood or bone marrow using standard procedures (8). Uncut placental DNA was purchased from Oncor (Gaithersburg, MD). The DNA concentration was determined by spectrophotometry, and the DNA samples were adjusted to a concentration of 50ng/ml in TE-buffer (10mM Tris Cl, 1mM EDTA, pH 8.0).

Polymerase Chain Reaction: PCR was performed with 200ng (4 μ l) genomic DNA under standard conditions in 50 μ l (50mM KCl, 10mM Tris Cl pH 8.3, 2.5mM MgCl₂, 0.01% (w/v) gelatin (Sigma), 250 μ M dNTPs (Boehringer), 0.225% Tween 20 (Sigma), 0.225% NP40 (Sigma), 1U AmpliTaq Polymerase (Cetus)).

The target gene in all experiments was the β -interferon gene and the reference (diploid) genes used were γ -interferon, and the HER-2/neu proto-oncogene. All priming oligonucleotides were 20 bases in length and the sequences for these PCR primers were taken from the published genomic sequences (9,10,11). The sequences of the oligonucleotides used are displayed in Table 1. Optimal concentrations for the priming oligonucleotides in the differential PCR were: 0.5 μ M for γ -interferon and neu, and

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Table 1: Sequences of the oligonucleotides used as PCR primers in this study. By convention, the priming oligonucleotide at the 5' end of the gene of interest is called the 5' primer, and that at the 3' end of the gene is designated the 3' primer.

Gene	5'primer	3'primer	Sequence region (Ref.)*
β -Interferon	GGCACAAACAGGTAGTAGGCG	GCCACAGGAGCTTCTGACAC	-40-130 (9)
γ -Interferon	AGTGATGGCTGAACTGTCGC	CTGGGATGCTCTTCGACCTC	4647-4731 (10)
neu	CCTCTGACGTCCATCATCTC	ATCTTCTGCTGCCGTCGCTT	2122-2219 (11)

* The oligonucleotide primers constructed for PCR correspond to the first 20 (5'-prime) and last 20 (3'-prime) bases within these reported sequences.

Lane	1	2	3	4	5	6	7	8	9	10	11
%placental DNA	0	10	20	30	40	50	60	70	80	90	100
ratio b/a	0.03	0.05	0.10	0.13	0.25	0.26	0.26	0.32	0.48	0.53	0.52

Figure 1: Differential PCR of dilutions of K562 DNA and placental DNA. This PCR was performed using ^{32}P labeled primers. Dilutions of placental DNA with K562 DNA were done in 10% steps. Shown is an autoradiogram of a 12% polyacrylamide gel. The bands marked with 'a' correspond to 84 bases amplified within the γ -interferon gene, and the bands marked with 'b' correspond to 170 bases amplified within the β -interferon gene. The ratios (b/a) were obtained using densitometry.

0.25 μM for β -interferon. The PCR proceeded for 27 cycles in a Perkin Elmer Cetus thermocycler under the following conditions: cycle 1: denaturing at 95° C for 5 minutes, annealing at 55° C for 1 minute, extension at 72° C for 1 minute; cycles 2-27: denaturing at 95° C for 1 minute, annealing and extension under conditions identical for cycle 1. One fifth of the PCR amplification products was electrophoresed on a 12% polyacrylamide gel and stained with ethidium bromide. The relative intensity of the PCR amplified bands was quantitated by densitometry of the photographic negatives of the ethidium stained gels. For quantification of radiolabeled PCR amplification products, the 3'-primers of both target and reference genes were labeled with ^{32}P using T4 kinase and γ -ATP (NEN) (12). The amplification products were electrophoresed on 12% polyacrylamide gels which were then dried and exposed to a Kodak film (XOMAT AR) for 30 to 60 minutes. Densitometry for all experiments was performed with a LKB densitometer. Results are expressed as the ratio of the area under the peak corresponding to the β -interferon divided by the area under the peak corresponding to the reference gene.

To test if sheared DNA would affect the differential PCR we sonicated placental DNA in TE (200ng/ μl). 15 μl were withdrawn

at different time points, 10 μl were electrophoresed on a 1% agarose gel stained with ethidium bromide, and 1 μl was used for differential PCR.

RESULTS

Optimization of Conditions for Differential PCR to Detect Hemizyosity at the β -Interferon Locus

Our choice for the reference gene in the differential PCR was γ -interferon located on chromosome 12 (4). We determined that differential PCR is able to detect homozygous gene loss. Using DNA from the cell line K562 no β -interferon band was visible after PCR whereas the γ -interferon band was present (Figure 1, lane 1). We then asked if the loss of one allele could be detected. 100ng of DNA from normal peripheral blood cells were mixed with 100ng of K562 DNA, thus mimicking the hemizygous condition at the β -interferon locus. Differential PCR amplification of this DNA mix was compared with that of placental DNA. Using equivalent concentrations of the primers for both the target and reference genes, little difference in intensity of the resultant PCR bands was detected (data not shown). However, by adjusting the ratio of the β -interferon primers to the γ -interferon primers

Table 2: Influence of the cycle number on differential PCR. 200ng K562 DNA, 100ng of K562 DNA mixed with 100ng placental DNA, and 200ng of placental DNA were subjected to differential PCR using radioactive labeled primers. After 20, 23, 27, and 30 cycles 5 μ l were removed and electrophoresed on a 12% polyacrylamide gel. The gel was dried and autoradiograms were taken. Shown is the ratio between the β - and the γ -interferon genes as measured by densitometry (see Materials and Methods section).

CYCLE NUMBER	0%	50% PLACENTA	100%
20	0.22	0.62	0.94
23	0.25	0.47	0.75
27	0.11	0.24	0.56
30	0.12	0.32	0.57

Figure 2: Differential PCR of DNA samples from 3 healthy blood donors. 100ng of normal DNA was mixed with 100ng of K562 DNA and subjected to 27 PCR cycles (lanes 1,3,5); as a control, 200ng of normal DNA was PCR amplified without the addition of K562 DNA (lanes 2,4,6). The ratios (b/a) were obtained using densitometry readings of a negative photograph of an ethidium bromide stained polyacrylamide gel. The ratios are (lane #): 0.30 (1); 0.54 (2); 0.30 (3); 0.58 (4); 0.20 (5); and 0.48 (6).

used in the differential PCR, the optimal primer concentrations giving the greatest difference between the hemizygous and the homozygous diploid state were found to be 0.5 μ M for γ -interferon, and 0.25 μ M for β -interferon. Thus the sensitivity of the system is dependent on the primer concentrations. We also tested if the ratio between the reference and the gene of interest was dependent on the number of PCR cycles. A differential PCR was performed using radioactive labeled primers. 5 μ l were removed after 20, 23, 27, and 30 cycles and electrophoresed on a 12% polyacrylamide gel. The results of this experiment are shown in Table 2. Though the β/γ ratios differ at different cycle numbers, the relative ratios between the homozygous diploid, hemizygous, and homozygous deleted conditions remain constant at each PCR condition. Thus the ability of differential PCR to discriminate allelic loss is independent of PCR cycle number. When gels were stained with ethidium bromide and band intensities detected by UV fluorescence, it was found that 27 cycles was optimal for detecting allelic loss (data not shown). Using these conditions, we tested the variability of the assay to detect hemizygosity by mixing genomic DNA from a number of normal blood donors with K562 DNA in a 1:1 ratio (50% mixture). The results of one experiment are shown in Figure 2. The ratio of the mixed samples was consistently lower compared to the normal DNA. When this was repeated with DNA from

9 healthy blood donors, the mean ratio of the 50% mixture was 0.32 (S.D. = 0.15), the mean ratio for the normal DNA samples was 0.82 (S.D. = 0.23).

The sensitivity of this assay to detect subtle levels of gene loss was tested by dilutions in 10% steps of K562 DNA with placental DNA. The results are shown in Figure 1 and reveal a consistent ability of differential PCR to detect gene dosage ratios of 3:2 (Figure 1, lane 7 and 8).

We then assessed the influence of target DNA fragmentation on differential PCR. Partially degraded placental DNA was further sonicated and aliquots were removed after certain time points (in 10sec intervals), analyzed by gel electrophoresis, and DNA from time points 0, 20, and 60 seconds were subjected to differential PCR. The results are shown in Figure 3. It is evident that no major changes in the ratio between β and γ -interferon were seen until DNA has been sonicated for one minute, at which time, the average DNA size is <300 base pairs. Thus, using correct controls, we feel that differential PCR is also suitable to analyze sheared DNA.

Differential PCR amplification of 64 CML cases

DNA from 64 cases with CML was subjected to differential PCR: 49 in chronic phase, and 15 in blast crisis. In each PCR run the following controls were performed: K562 DNA alone to test possible contamination, DNA from a normal blood donor, and the 50% mixture thereof with K562 DNA. Only 1 of 64 CML cases (CML case #25) revealed a lower ratio between the reference gene and β -interferon gene (Figure 4A). This finding was corroborated by a differential PCR using primers within the neu oncogene as the reference gene (Figure 4B). Here also this patient's DNA revealed a lower β -interferon/neu ratio. This patient (patient # 25) was in blast crisis and had no apparent cytogenetic abnormality involving chromosome 9.

To test for possible inhibition of in vitro PCR amplification due to blood contaminants (13), we applied differential PCR on a mixture of 100ng of the patient DNA with 100ng placental DNA and compared this with differential PCR of 200ng of the patient DNA. There was no inhibition of PCR since the mixture of the patient and placental DNA showed the expected increase of the ratio β/γ -interferon, whereas if the PCR was inhibited, reduced amplification would have been seen (data not shown) (13). Furthermore, the difference in intensities between the β and γ bands was not due to severe DNA shearing since the patient DNA used was >10 kb. Unfortunately, confirmation of allelic loss at the β -interferon locus by Southern hybridization using DNA from patient #25 could not be performed since only 1 μ g of genomic DNA from this patient remained after the PCR analyses.

DISCUSSION

Polymerase chain reaction has been used in recent years for the analysis of oncogene abnormalities such as point mutations of the ras genes (12,14), and specific chromosomal translocations (15). The advantages of PCR based techniques are that only miniscule amounts of DNA are necessary, that PCR can be performed on paraffin embedded tissue, and that PCR based techniques are simple and fast. It has been reported that PCR can be used to detect homozygous germline deletions of the α globin and the Duchenne muscular dystrophy loci (16,17). We have recently reported that gene amplification can also readily be measured using a modification of PCR called differential PCR (7). Here we show that allelic loss leading to hemizygosity can

Figure 3: Differential PCR using sonicated DNA. DNA was sonicated (see Materials and Methods section), and aliquots were removed in 10sec intervals. Figure 3A shows an ethidium bromide stained 1% agarose gel. Lane 1: Marker DNA ϕ X174 restricted with HaeIII. Lane 2: DNA partially sheared by vigorous shaking. Lane 3: 10 sec, lane 4: 20 sec, lane 5: 30 sec, lane 6: 40 sec, lane 7: 50 sec, and lane 8: 60 sec sonicated. Figure 3B: 1 μ l of sonicated DNA was subjected to differential PCR using primers for β and γ -interferon for 27 cycles and electrophoresed on a 12% polyacrylamide gel stained with ethidium bromide. Lane 1: Unsonicated DNA (ratio b/a: 0.96), lane 2: 20 sec sonicated DNA (ratio b/a: 1.0), and lane 3: 60 sec sonicated DNA (ratio b/a: 0.78). Thus, sonication of DNA to <300 base pairs has no major influence on the ratio b/a.

be detected with differential PCR. The assay is based on quantification of the ratio between a reference gene and the gene of interest and can readily detect subtle changes in the target:reference gene ratios, e.g. ratios of 2:1 and 3:2. The success of this assay was dependent on the careful adjustment

of the PCR conditions. Our current differential PCR conditions are capable of analyzing DNA isolated from minor cell populations by cell sorting or microsurgery. Furthermore, the sensitivity of our assay is not affected by significant fragmentation of DNA as commonly seen in DNA extracted from paraffin

Figure 4: Differential PCR of DNA from various patients with CML. 4A: shown are CML patients # (ratio b/a) lane 1: # 3 (0.68); lane 2: # 25 (0.35); lane 3: # 49 (0.57); lane 4: # 57 (0.61); and lane 5: # 64 (0.74). As controls, a 1:1 mixture of normal blood with K562 DNA was performed and is shown in lane 6 (0.43), and normal blood alone is shown in lane 7 (0.76). The band marked with 'a' corresponds to 84 bases amplified with γ -interferon specific primers, and the band marked with 'b' corresponds to the 170 bases amplified within the β -interferon gene. 4B: shown are CML patients # (ratio b/a) lane 1: # 1 (1.47); lane 2: # 3 (1.0); lane 3: # 24 (1.3); lane 4: # 25 (0.71); lane 5: # 29 (1.3). Lane 6 represents a 1:1 mixture of normal blood DNA with K562 DNA (0.67), and lane 7 shows amplification of normal blood DNA alone (1.15). For this experiment, primers specific for the neu oncogene were used as a reference revealing a 98 base pair fragment (band 'a'). The band marked with 'b' represents the 170 base pair fragment amplified within the β -interferon gene. Thus patient CML # 25 revealed a lower ratio using two different genes as reference genes (γ -interferon and neu oncogene).

embedded tissues (Figure 3). These capabilities make differential PCR superior to conventional Southern hybridization for the analysis of hemizyosity in human tumor samples.

In developing a PCR-based technique for detection of allelic loss we took advantage of the homozygous loss of the α and β -interferon genes in the human CML cell line K562 (1). The presence of deletions at this locus in such a CML blast crisis cell line suggests that loss of a β -interferon gene or a linked gene plays a role in the progression of CML to blast crisis. Our data show that the loss of a β -interferon allele occurred in one of 15 patients with blast crisis and none of 49 patients in chronic phase. Though it is possible that deletions at this locus may play a role in the tumor progression of a small number of CML patients, it is not likely that this role is as important as that in acute lymphocytic leukemia (1), where 28% of patients exhibit some perturbation at the β -interferon locus. We do not believe that dilution with normal blood cells can account for the low frequency of allelic loss of the β -interferon gene seen in our series, since

CML is a clonal disease that affects all bone marrow lineages (18). Thus, the majority of cells in the blood and bone marrow carry the typical genetic and molecular abnormalities of the disease.

ACKNOWLEDGEMENTS

We thank Dr. Roy Frye for helpful discussions, and Drs. A.A. Sandberg and Rodman Morgan for the CML patient samples. This research was supported in part by grants from the Deutsche Forschungsgemeinschaft (Ne 310/4-1), Deutsche Krebsgesellschaft Berlin (A.N.), NIH grant:R01-CA49240-01 (E.L.), and the Rodney Foppiano Memorial Fund. E.L. is a Leukemia Society Special Fellow.

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