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Chromosome 7 long arm deletion breakpoints in preleukemia: mapping by pulsed field gel electrophoresis

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ABSTRACT

Chromosome 7 long arm deletions (7q-) are recurring chromosome abnormalities in leukemic bone marrow cells. In four patients we have previously localized the breakpoints in band 7q22 between the erythropoietin (EPO) and plasminogen activator inhibitor type 1 (PLANH1) genes that map 3 cM apart. The proalpha2(I)collagen (COL1A2, in band 7q22) and T cell receptor beta chain genes (TCRB, in band 7q35) have been found undeleted in one patient with an interstitial deletion. Pulsed field gel electrophoresis was used to map the breakpoints more accurately in two patients with a 7q- chromosome. The results suggested that lymphocytes and granulocytes give identical restriction patterns with several enzyme-probe combinations, and that a breakpoint possibly was within 195 kb of EPO in one patient but not in another. The gene order cen-COL1A2-EPO-breakpoint-tel was suggested but physical linkage between COL1A2 and EPO was not found. A new putative TCRB restriction fragment length polymorphism or inherited methylation site was detected.

INTRODUCTION

Monosomy for chromosome 7 and partial deletion of the chromosome 7 long arm (7q-) are among the common recurring clonal chromosome abnormalities observed in bone marrow cells in patients with acute nonlymphocytic leukemia (ANLL) or myelodysplastic syndrome (MDS) (1,2). The biological basis of this phenomenon is not understood. It might involve the loss of a gene as in retinoblastoma (3), the rearranged fusion of two genes as in translocation (9;22) resulting in the formation of a Philadelphia chromosome (4), or the dysregulation of a gene as in lymphoid diseases (5). The loss of a chromosome 7 long arm has been associated with susceptibility to infections, a granulocyte locomotor defect, rapid progression of the disease, and poor response to therapy (6-9). One step toward the understanding of these features is to determine the 7q- chromosome breakpoint(s) in molecular terms.

Molecular mapping studies have indicated that in at least some patients and deletion types a chromosomal breakpoint involves a narrow region in band 7q22. In 4 patients the breakpoint was localized between the erythropoietin

(EPO) and plasminogen activator inhibitor type 1 (PLANH1) genes mapping 3 cM apart, a distance comprising less than 2% of the total length of chromosome 7 (10-12). In one patient the proalpha2(I)collagen (COL1A2) gene in band 7q22 and a distal segment including the T cell receptor beta chain (TCRB) gene in band 7q35 were retained (13). EPO and COL1A2 are tightly linked with 0 cM genetic distance (11).

Pulsed field gel electrophoresis (PFGE) techniques are novel methods for the separation of very large DNA fragments produced by restriction enzymes for rarely occurring sequences (14-16). PFGE analysis has been used to detect deletions in patients with Duchenne muscular dystrophy and to produce long range restriction maps in a few genomic regions, e.g. around the cystic fibrosis and Duchenne muscular dystrophy genes (17,18). Field inversion gel electrophoresis has also been used to enrich selected genomic regions in the cloning of new segments within a hundred-kilobase distance from a probe (19).

This study had three objectives: first, to develop methods for the mapping of 7q- breakpoints by PFGE, second, to determine more accurately the breakpoints in two patients, and third, to study the physical distance of the tightly linked COL1A2 and EPO loci preserved in the 7q- chromosomes.

MATERIALS AND METHODS

Patients

Two patients with a 7q- chromosome, 2 patients with other chromosomal abnormalities, and altogether 18 control subjects were studied. A diagram of the 7q- chromosome in patients 1 and 2 based on previous results (10,13) is shown in Figure 1.

Patient 1, a female aged 77, had had polycythemia vera and had received chemotherapy and radiophosphorus. At the age of 72 her disease turned to MDS. At the time of the study her bone marrow cytology was compatible with refractory anemia with excess of blasts (RAEB) as classified according to the proposal of the French-American-British group (20). The bone marrow karyotype was interpreted as 46,XX,del(7)(pter->q21::q32-35->qter)=16 (21).

Patient 2, a male aged 82, had developed 2 years earlier a primary MDS compatible with FAB classification RAEB. His bone marrow karyotype was 46,XY,t(2;13),del(7)(pter->q22::q34->qter)=20.

Patient 3, a male with no history of leukemogenic exposure, developed myelofibrosis at the age of 57. At diagnosis one clone with the karyotype 46,XY,del(13)(q12q14) was detected in phytohemagglutinin-stimulated blood

mitoses in addition to normal cells. A blood sample for this study was obtained 3 months after diagnosis.

Patient 4, a 58 years old male, had no history of leukemogenic exposure. At the age of 57 he developed MDS, and a year later ANLL. FAB classification was not possible due to fibrosis of the bone marrow. Blood cultures made without any mitogen at the leukemic stage revealed the karyotype 46,XY=5/46,XY,-7,+t(1;7)(cen;cen)=5/47,XY,-5,+6,-7,+t(1;7),+mar=1/48,XY,-7, +t(1;7),+13,+21=1.

Samples and DNA preparation

White blood cells were recovered from fresh blood samples (20 to 50 ml) by removing erythrocytes with dextran sedimentation and used unfractionated from healthy subjects (22). Granulocytes and mononuclear cells were further fractionated from patient samples by Ficoll (Pharmacia, Uppsala, Sweden) gradient centrifugation (22). Freshly isolated cells were embedded in 0.5% low-melting-point agarose (Bethesda Research Laboratories, Gaithersburg, MD) at $10x10^6$ cells/ml. The gel blocks were allowed to set at 0^oC, transferred to a solution containing 0.25 M EDTA pH 8.0, 1% sarcosyl (Fluka, Buchs, Switzerland), and 2 mg/ml proteinase K (Merck, Darmstadt, Germany), and incubated for 48-60 h at 50^oC. A minimum of 10 ml of the solution was used per 15 million cells. The blocks were washed twice with TE buffer (23) pH 8.0 and twice with TE containing 40 ng/ml phenylmethylsulphonylfluoride (Sigma, St.Louis, MO) at 50^oC, and stored in TE or 0.5 M EDTA pH 8.0 at 4^oC. For restriction enzyme digestion, a block ($2x5x6\ mm^3$) was equilibrated with the appropriate restriction enzyme buffer (REB) (24), and incubated for 4 h in fresh REB (100 ul) with 10-20 units of the restriction enzyme. The samples were electrophoresed in 1% agarose gels. Saccharomyces cerevisae chromosome preparations and bacteriophage lambda multimers were used as size markers (14). The kb-estimates given are approximate and varied by 5 to 10% from one blot to another.

Pulsed field gel electrophoresis

The electrophoreses were performed in workshop-made tanks designed according to the contour-clamped homogeneous electric field principle (16). Workshop-made timers were used as switching units (25). Temperature control was arranged with thermostatic flow heater and cooler units (Grant, Cambridge, England). The electrophoreses were made in 0.25xTBE buffer (23) at $12-14^oC$. The switching intervals varied from 20 to 900 s, voltage from 80 to 150 V, and total electrophoresis times from 1 to 6 days in different experiments.

Table 1. Properties of the DNA probes.

Gene	Location	Probe	Type	Reference
COL1A2	7q21.3-q22.1	NJ-3	genomic DNA	26
EPO	7q22	EPO	cDNA	27
PLANH1	7q21.3-q22	PAI1	cDNA	28
MET	7q31	MetH	genomic DNA	29
TCRB	7q35	TCRB	cDNA	30

The gels were stained with ethidium bromide, photographed, and irradiated for 5 min on a Macrovue transilluminator (LKB, Bromma, Sweden). Denaturation, neutralization, capillary transfer of DNA to nitrocellulose filters with blotting times of 2 to 3 days, and hybridization to DNA probes were done as described (23). The DNA probes (Table 1) were labeled with ^{32}P by nick-translation or oligolabeling using Amersham (Buckinghamshire, England) or Pharmacia kits.

RESULTS

Properties of DNA from different white blood cell fractions

An ethidium bromide stained PFGE gel with undigested DNA revealed that all samples were of very high molecular weight whether prepared from white blood cells, blood mononuclear cells, or granulocytes. Equal amounts of restriction enzymes were used for all DNA types in restriction digestions. Similar digestion and hybridization results were obtained in most cases from DNA derived from separated cell fractions or unseparated white blood cells. The exceptions are described below.

Search for physical linkage between EPO and COL1A2

DNA derived from unfractionated white blood cells from a healthy subject was used in the mapping experiments with 6 restriction enzymes. The COL1A2 sequences mapped to MluI and NotI fragments of approximately 1500 and 1400 kb, respectively, and to a BssHII fragment of 840 kb. In different blots, 2 or 3 SacII fragments in the size range 450 to 700 kb, and a 420 kb PaeR7I fragment with varying numbers of smaller fragments (<200 kb) were detected. SfiI produced fragments of <200 kb only. In contrast, the EPO probe detected different fragments the largest of which was a MluI fragment (approximately 480 kb).

To confirm that COL1A2 and EPO mapped to different fragments and to rule out differences in digestion on different blots, a single blot was used in

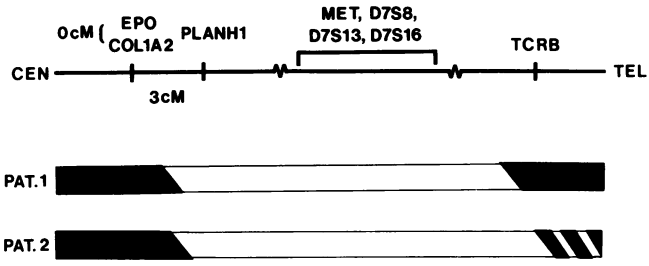

Figure 1. Diagram of a section of the chromosome 7 long arm showing the extent of the deletion in patients 1 and 2. Black, preserved segment; white, deleted segment; striped, extent of deletion unknown.

repeated hybridization experiments with the probes. No hybridization bands were shared by the tightly linked COL1A2 and EPO.

Search for the deletion breakpoint in 7q- patients

Our previous results had indicated that granulocytes but not lymphocytes were derived from an abnormal stem cell clone with a 7q- chromosome in patients with MDS or ANLL (13). This was confirmed by conventional RFLP analysis of TaqI-digested DNA blocks from mononuclear cells (lymphocytes and monocytes) and from granulocytes. Probe MetH was used. Patients 1 and 2 were heterozygous for the MetH/TaqI RFLP, and mononuclear cell-derived DNA thus revealed two allelic bands. In the granulocyte-derived DNA one of the allelic bands was very weak indicating deletion of the chromosomal segment containing MET (Figure 2 A).

PFGE blots were used in hybridization experiments with probes for EPO, COL1A2, and TCRB preserved in the 7q- chromosomes (Figure 1). Restriction digestions were done with enzymes that produced the longest fragments in control DNA. The COL1A2 probe detected only one fragment of the expected size on blots containing DNA digested with NotI, MluI, or BssHII (Figure 2 B).

The TCRB probe detected one fragment in the NotI and SacII digested samples from patients 1-3 (not shown, patient 4 was not studied) and BssHII digested samples from patients 2 and 3. Two fragments (approximately 1070 kb and a weaker 810 kb fragment) were detected with the TCRB probe in BssHII digested mononuclear cell and granulocyte samples from patients 1 and 4 (Figure 2 C). This pattern suggested a RFLP rather than a molecular rearrangement.

The EPO probe detected one fragment of the same size as in controls in both NotI digested fractions from patients 1 and 3 (patient 4 was not studied). In the mononuclear cell fraction of patient 2 there was a strongly

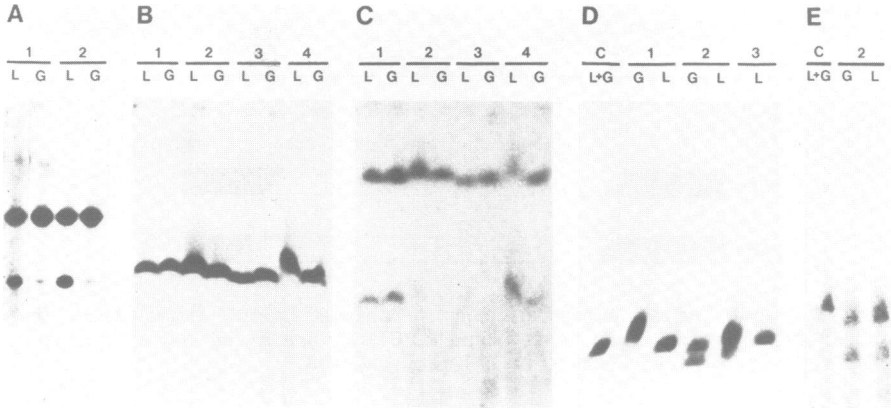

Figure 2. Search for the 7q- breakpoints. DNA derived from separated mononuclear cells (L) and granulocytes (G) were studied. A, samples from patients 1 and 2 were digested with TaqI, electrophoresed for 30 V overnight, and hybridized to probe MetH. One of the allelic bands in the granulocyte lane was very weak, indicating that most of the granulocytes had a 7q- chromosome. B, mononuclear cell-derived and granulocyte-derived DNA from patients 1-4 was digested with BssHII and hybridized to a COL1A2 probe. One fragment was detected in all samples, suggesting that the deletion breakpoint was not within this fragment. The PFGE was at 145 V for 39 h with a pulse time of 120 s. C, samples from patients 1-4 were digested with BssHII and hybridized to a TCRB probe. Two bands were detected in both the L and the G lanes of patients 1 and 4, suggesting a BssHII/TCRB RFLP. D, samples from patients 1-3 and a healthy control (C) were digested with NotI and hybridized to an EPO probe. Two fragments were detecetd in the G lane of patient 2 but one strong in the L lane, suggesting that the deletion breakpoint mapped to this fragment. The PFGE was at 120 V for 42 h with a pulse time of 120 s. E, SacII digested samples from patient 2 and a control hybridized to the EPO probe revealed two equally strong fragments in the G lane, whereas the smaller fragment was much weaker in the L lane. The result is compatible with a rearrangement due to 7q- deletion. The PFGE was at 130 V for 63 h with 30 s pulses.

hybridizing band with some smear in the direction of lower molecular weights, and in the granulocyte fraction two equally strongly hybridizing bands, one of them of the expected size (195 kb) and the other approximately 40 kb smaller (Figure 2 D). A repeated experiment gave similar results; notably, no evidence for a rearrangement was obtained in patient 1. Two fragments were also detected in a SacII digested granulocyte sample from patient 2, whereas his mononuclear cell sample showed a strongly hybridizing fragment of normal size and a weaker rearranged fragment (Figure 2 E). Both samples from patients 1 and 3 showed only one strongly hybridizing fragment (not shown).

Frequency and inheritance of the suggested TCRB RFLP

The TCRB/BssHII hybridization pattern (Figure 2 C) could be explained as

a RFLP. Blood DNA from a control subject with two fragments was digested with different amounts of BssHII (range 0.5 to 30 U) to study partial digestion patterns. The digestion pattern was constant over this range of enzyme concentrations (not shown). In a study of 10 unrelated control subjects the 1070 kb TCRB/BssHII fragment was detected in all 10, and an additional 810 kb fragment in 4. The frequency of the 810 kb band was thus 6/14 (including 4 patients). The relatives of 2 subjects with both fragments were studied. In one family both fragments were observed in both parents of the proband. In another family both parents had both fragments and they were also detected in all 4 children (not shown). These results were consistent with Mendelian inheritance.

DISCUSSION

Our previous results had suggested that a relatively short breakpoint region (3 cM) in band q22 was involved in the 7q- deletion in 4 patients (10). In this study an attempt was made to localize the breakpoint more accurately by PFGE directly by studying separated blood cells. DNA used in PFGE experiments is usually prepared from cultured lymphoblasts or unfractionated blood cells and the results have suggested that there are differences in methylation between these DNA sources (17,19). In the present study standard protocols were used in the preparation of DNA from freshly fractionated lymphocytes and granulocytes, and very high molecular weight DNA was obtained in all cases. DNA prepared from both cell types gave similar restriction patterns with several enzyme and probe combinations. Slight variation was observed in the position of hybridization bands (Figure 2 B-E). This was probably due to differences in the amount of DNA per lane (31). In the mapping experiments some restriction enzymes (MluI, SacII, SfiI) repeatedly produced multiple hybridizing bands that probably reflected the presence of digestion-resistant sites, perhaps due to methylation (not shown). The presence of RFLPs was, however, not ruled out.

The close linkage between EPO and COL1A2 (0 cM genetic distance) offered a possibility to study the physical relationship between these genes by PFGE. Probes for EPO and COL1A2 detected fragments of different sizes, and no shared fragments were detected when a single blot was hybridized successively to both probes. Assuming that there were no inversions or translocations in the 7q- chromosome, the detection of a rearranged band in patient 2 suggested a probable order for these genes: centromere-COL1A2-EPO-breakpoint-telomere. Experiments are in progress to construct a large-scale physical map including

these and other tightly linked sequences (12) from this region.

Our previous results had indicated that the 7q- chromosome was present in blood granulocytes but not in lymphocytes in patients with MDS or ANLL (13). This was confirmed by RFLP analysis in the samples prepared for PFGE analysis from patients 1 and 2 (Figure 2 A). Thus, if the breakpoint were located in the same restriction fragment as the probed, preserved sequence, then one would expect to find one normal and one rearranged fragment in the granulocyte sample whereas the rearranged band should be weak or missing in the mononuclear cell sample. The three enzymes that produced the longest restriction fragments were used to screen for rearranged bands in the patient samples.

Only expected fragments were detected by a COL1A2 probe. The most probable interpretation was that the chromosomal breakpoints in patients 1 and 2 were not located within the restriction fragments containing COL1A2. The EPO probe repeatedly detected two fragments in the granulocyte DNA but only one fragment in the mononuclear cell DNA when hybridized to NotI digested samples from patient 2 (Figure 2 D). This pattern was compatible with the expected rearrangement pattern caused by the deletion. These results suggested that the deletion breakpoint in patient 2 was within 195 kb of EPO. An alternative explanation is that the difference between cell fractions was caused by differences in digestion due to e.g. methylation. This was unlikely for several reasons: first, differences between mononuclear cells and granulocytes were not observed in other patients, second, two bands were never detected in whole blood samples from 3 healthy subjects, and third, the unexpected band was detected by two different restriction enzymes (NotI and SacII, Figure 2 E). Patient 1 had only one band in both cell fractions, suggesting that the deletion breakpoints in patients 1 and 2 were different. This interpretation was not incompatible with our previous results (10, 13). Definitive evidence that the rearranged band in patient 2 indicated the deletion requires cloning of the breakpoint.

The TCRB probe detected an unexpected band in patients 1 and 4 in BssHII digested DNA samples (Figure 2 C). The unexpected band was of the same size (810 kb) in both patients, and similar in strength in both mononuclear cell and granulocyte fractions in patient 1. Patient 1 had a 7q- chromosome whereas patient 4 had monosomy for the whole chromosome 7 long arm. In patient 4 the unexpected band was present also in the granulocyte lane, although very weak. This could be due to the presence of both normal cells and leukemic cells with monosomy 7, as suggested by the chromosome study.

Altered fragments were not detected in blots made with other restriction enzymes. These observations suggested a RFLP rather than a molecular rearrangement. The digestion pattern was constant over a wide range of enzyme concentrations, suggesting that the 810 kb fragment was not caused by partial digestion. The 810 kb fragment was found in 6/14 unrelated subjects and the family studies were consistent with Mendelian inheritance. The 1070 kb band was detected in all individuals. The study of the allele systems and nature of this putative RFLP is under way; it might alternatively be due to an inherited methylation site (32). At least one RFLP has previously been detected by PFGE (MET/NotI) (31).

In conclusion, PFGE studies on blood cells from patients with preleukemia and with or without 7q- chromosomes as well as healthy subjects suggested that (i) fractionated white blood cells can be used as a source of DNA for PFGE studies and they have similar properties, (ii) one of the deletion breakpoints appeared to be located within 195 kb of EPO in one patient but not in another with a 7q- chromosome, (iii) COL1A2 is probably located more proximally than EPO in 7q22, and the two genes map to different rare-cutter restriction fragments, and (iv) a new TCRB/BssHII RFLP or inherited methylation site was detected.

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