Haematologica 1998; 83:403-407 original paper

Microsatellite analysis in childhood acute lymphoblastic leukemia

Gigliola Reato,* Giuseppe Basso,° Maria Cristina Putti,# Alessandro Cignetti,* Anna Guarini,* Robin Foa*

**Dipartimento di Scienze Biomediche ed Oncologia Umana, University of Turin; °Dipartimento di Scienze Pediatriche, University of Turin; #Dipartimento di Pediatria, University of Padua, Italy*

Abstract

Background and Objective. **Genetic alterations, including genomic instability, represent possible steps towards a malignant transformation. One approach to delineate replication errors in cancer cells is to determine alterations of microsatellites that are short tandem repeat sequences dispersed throughout the human genome. We have investigated whether genomic instability may be a possible event in the leukemogenic process by evaluating the pattern of instability in 41 cases of childhood acute lymphoblastic leukemia (ALL).**

Materials and Methods. **Eighty-two samples of genomic DNA (41 at diagnosis and 41 at remission) were analyzed by PCR with microsatellite markers chosen on five different chromosomes (2, 10, 11, 13, 18) known to be frequently involved in tumors of various origins. Since deletions of the short arm of chromosome 12 are relatively common in children with ALL, we also analyzed one region flanked by the microsatellite marker D12S308 on 12p. This area encompasses a genetic locus which contains the putative suppressor gene KIP1.**

Results. **A pattern of MI at one or two loci on different chromosomes could be documented in 4 of the 41 cases analyzed (9.7%). Three were common ALL and 1 was a T-ALL. One case showed two concomitant sites of instability, while 1 revealed two additional bands by using simultaneously microsatellite markers D2S123 and D18S58.**

Interpretation and Conclusions. **These results indicate that genetic instability of microsatellite repeat sequences occurs in a proportion of childhood ALL. Mismatched repair errors documented in hereditary and sporadic solid tumors may thus be involved in hematological malignancies. While in such cases the pattern of genomic instability appears indicative of a mutator phenotype and of a potential predisposition towards a leukemic transformation, other genomic loci close to cytogenetic and molecular alterations known to occur in ALL need to be investigated in depth in cases with an apparently non mutated phenotype.**

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Key words: acute lymphoblastic leukemia, genomic instability, microsatellite analysis

Correspondence: Dr. Gigliola Reato, Dipartimento di Scienze Biomediche ed Oncologia Umana, Sezione Clinica, via Genova 3, 10126 Torino, Italy. Phone: international +39-11-6637230 • Fax: international +39-11-6637238.

Accumulation of multiple genetic abnormalities causing activation of oncogenes or loss of function of tumor suppressor genes has been described in many human cancers. To develop a tumor, several specific mutations in the same cell are necessary and probably are based on disturbances in the stability of the genome. These alterations have been shown to involve short repeated nucleotide sequences called microsatellites distributed within the human genome.[1] Microsatellite instability (MI) at multiple genetic loci, initially reported in sporadic colon cancer and in hereditary nonpolyposis colorectal cancer (HNPCC),[2] appears to be a novel molecular mechanism in cancerogenesis associated with a defect of mismatch repair genes. These include five different genes: hMSH2, hMSH3 (DUG), hMLH1, hPMS1and hPMS2 which map on different chromosomes.[3-6] Besides colorectal cancers, MI has been described in a variety of sporadic tumors including breast, endometrial, ovaric, gastric, bladder and lung cancer.[1,7] There is also some evidence to suggest that MI may be detected in hematological neoplasies.[8-10] Wada *et al*.[11] suggested that MI may be a frequent feature in the evolution of chronic myeloid leukemia (CML) from chronic phase to blast crisis. On the contrary, another study[12] reported that in the majority of cases there is no consistent difference in microsatellite sequences between CML in chronic phase and in blast crisis. Since genetic instability could be a common mechanism involved in neoplastic transformation, in the present study we have examined the pattern of MI in 41 cases of childhood acute lymphoblastic leukemia (ALL) comparing DNA at diagnosis and at remission from the same patients. MI was identified using specific primers for each locus and visualized by autoradiography. Moreover, since cytogenetic studies of childhood ALL[14] have identified different chromosomal abnormalities, including deletions of the short arm of chromosome 12, we have extended the analysis to the 12p13, the region which is flanked by the microsatellite marker D12S308 (Figure 1). This region contains the KIP1 gene, a potent inhibitor of cyclin-dependent kinases, which may be a negative regulator of cell proliferation.[10] In our study, we have screened 6 microsatellite markers, D2S123, D10S197, D11S904, D13S175, D18S58 and D12S308, on different chromosomes to deter-

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mine a possible pattern of genetic instability in childhood ALL. MI was found in 4 of the 41 cases studied, suggesting that in the latter, this molecular event could be associated to loss of fidelity in DNA replication.

Materials and Methods

Samples

The study was conducted on DNA from bone marrow samples collected at diagnosis and at complete remission from 41 cases of children with ALL. According to the immunophenotypic profile at presentation, 14 were pre-B ALL, 22 were common ALL and 5 were T-ALL. The state of complete remission was based on standard clinical and hematologic criteria. Genomic DNA was extracted by phenol-chloroform standard procedure followed by ethanol precipitation.

Microsatellite instability

DNA were amplified by PCR at five microsatellite markers, D2S123, D10S197, D11S904, D13S175 and D18S58, which have shown a relatively high degree of genomic instability in colorectal tumors and in other different forms of cancer. In addition, we have extended the analysis at the D12S308 microsatellite marker on chromosome 12p. The primer sequences for microsatellite markers were obtained from the *Genethon Microsatellite Map Catalogue of Human Genome Research Center of Evry* (France). PCR was performed using 1× buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.01% w/v gelatin), 200 µM concentrations of each deoxynucleotide triphosphate, 25 pmol of each primer (5 Prime→3 Prime), 2.5 U AmpliTaq polymerase (Perkin Elmer-Cetus, Norwalk, CT) and 0.5 µg of genomic DNA. Eight µCi of (^{35}S) labeled-dATP were incorporated in a volume of 20 µL of reaction mixture. Samples were processed through 27 cycles of amplification consisting of 30 sec at 94° C, 75 sec at 55°C, 15 sec at 72°C followed by a final extension at 72°C for 6 min after the last cycle. PCR products were denatured by 95% formamide and electrophoresed on 7 M urea polyacrylamide gel for 2 h at 59 W. Subsequently, the gels were fixed in 10% acetic acid, dried and exposed to Amersham film (Buckinghamshire, UK) for 7 days.

MI was defined as one or more bands present in addition to the pattern of bands amplified from remission bone marrow samples from the same patient. Furthermore, we have considered a case positive for MI when a variation of the radiographic signal intensity of the PCR products comparing DNA extracted at diagnosis and remission of the same patient was visible.

Results

Six microsatellite markers D2S123, D10S197, D11S904, D13S175, D18S58 and D12S308 from six different chromosomes loci were amplified by PCR using specific primers for each chromosome locus. In 4 of the 41 cases analyzed (9.7%), we found differences in the pattern of microsatellite sequences, comparing the DNA at diagnosis and at remission from the same individual. Three were common ALL and 1 was a T-ALL. One case showed two bands in addition to the bands amplified from bone marrow samples at remission on microsatellite marker D11S904, while 1 of the 4 positive cases (#2) showed two additional bands by simultaneously using microsatellite markers D2S123 and D18S58. The types of alterations were similar to those observed in sporadic colorectal cancer[15] and included a characteristic pattern of new fragments of variable size. In contrast, 2 cases showed an increase of radiographic signal intensity visible in the pattern of DNA extracted from bone marrow at remission compared with matched DNA at diagnosis. Figure 2 shows the electrophoretic alterations in 2 positive cases from microsatellite repeat sequences on chromosomes 2 (D2S123), 11 (D11S904) and 18 (D18S58), while the results of PCR products amplified on chromosome 12p are represented in Figure 3. In this locus, the microsatellite pattern was visible without additional bands, but the intensity ratio between DNA at diagnosis and at remission from the same patient was altered. This molecular event could be the result of a genetic imbalance such as loss of heterozygosity (LOH) on constitutional alleles. The phenomenon, which has previously been described in various types of tumors,[16] suggests the presence of a suppressor gene within the chromosome region. We could demonstrate a LOH at a single locus on chromosome 12p using the D12S308 microsatellite marker in 2 of the cases analyzed.

The variation of radiographic signal intensity of the PCR products was also evaluated by densitometric analysis. The values obtained showed an increase of intensity visible in the pattern of DNA extracted from

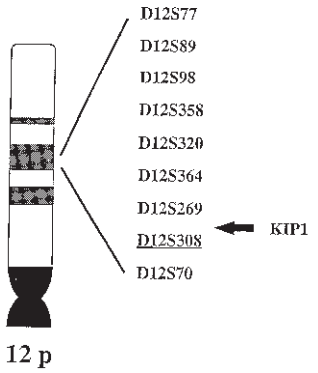

Figure 1. Schematic representation of the microsatellite marker on chromosome 12p derived from the Généthon Microsatellite map (see ref. #13). The arrows refer to the chromosomal region flanked by the microsatellite marker which includes the KIP1 gene, frequently deleted in childhood ALL.

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bone marrow samples at remission compared with matched DNA collected at diagnosis (Figure 4).

The positive cases were confirmed by two or three independent PCR reactions.

Discussion

In the present study we have investigated the pattern of instability of microsatellite repeat sequences in bone marrow samples of 41 children with ALL. Using six microsatellite markers on six different chromosome loci we have found evidence of MI in 4 of the 41 cases examined (9.7%). Although the number of microsatellite repeat sequences analyzed is relatively limited, these results indicate that somatic instability may be present in ALL. Several studies[2-6,17] have demonstrated that for many tumors such changes are the result of genomic instability which causes numerous nonspecific genetic alterations, such as errors in the replication of DNA. Strand *et al*.[18] reported that repetitive DNA destabilization in yeast is associated with a reduction in heteroduplex repair efficiency which leaves errors uncorrected. This has been associated with a defect of mismatch repair genes in both the hereditary and sporadic forms of human colon cancer.[2,19] It is possible that a similar mechanism may be involved in hematologic malignancies, although this has not yet been demonstrated. An approach to delineate replication errors in leukemic cells is based on the analysis of MI as an indirect indicator of defects in DNA repair mechanisms. The frequency of mutational events is not known in the genome of these cells; however, it is possible to demonstrate critical sites in the DNA that present a certain predisposition to replication errors, defined by molecular changes of repeat sequences. In our study, we have utilized microsatellite markers which are known to have a high degree of instability in colorectal cancer and in other tumors.[2,7] Using such primers, we could identify 1 case positive for genomic instability at a single locus and 1 case simultaneously positive for two chromosomal loci. We believe that the frequency of MI, even at a single locus, is informative and probably due to the fragility of genomic sites. This same approach was utilized by Gartenhaus *et al*.[20] who reported an allelic loss in at least one locus in 31% of chronic lymphocytic leukemia samples. The single locus approach was utilized in one study on non-small cell lung cancer[21] and not used in another[22] in which a multiple locus involvement was required.

A high incidence of mutations, including activation of oncogenes or inactivation of tumor suppressor genes with or without LOH in different chromosomes, has been reported in the genome of ALL cells. The loss of genetic material, detected as LOH of the microsatellite sequences D12S308, reflects a critical region that contains a possible tumor suppressor gene.[10] Thus, the variation of autoradiographic signal intensity can be considered an alternative indicator of chromosome

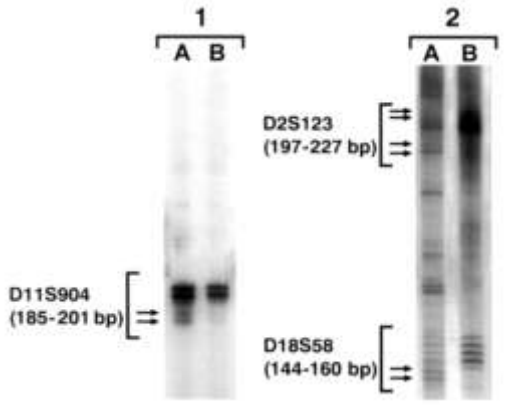

Figure 2. Alterations of the microsatellite repeat sequences D11S904, D2S123 and D1S558 in positive cases (#1 and #2). The arrows show new smaller fragments observed in DNA samples at presentation compared with matched DNA at the time of complete rernission from the same patient. Lane A and B represent bone marrow samples at diagnosis and at remission. Case #2, analyzed by multiplex PCR, shows alterations of both the D2S123 and D1S558 microsatellite markers.

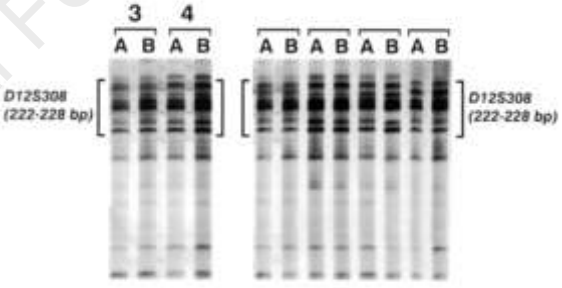

Figure 3. Representative autoradiographs of microsatellite analysis of cases #3 and #4 showing LOH at the KIP1 gene; bone marrow samples at diagnosis and at remission were amplified with the microsatellite marker D12S308. Lanes A and B represent bone marrow samples at diagnosis and at remission. A panel of four negative cases is presented in the right panel of the figures.

microdeletions in general representative of a pattern of genomic instability.[23-25] This was the picture observed in 2 further cases of our series (#3 and #4) in which the reduced signal intensity compared to that observed in the remission samples reflected an imbalance originating from microdeletions on chromosome 12p. Stegmaier *et al*.[25] did not detect mutations in leukemia cells of childhood ALL patients with LOH on 12p by exon analysis of KIP1 gene. However, it is possible that genetic point mutations could lead to the decreased expression of the KIP1 gene product, as sug-

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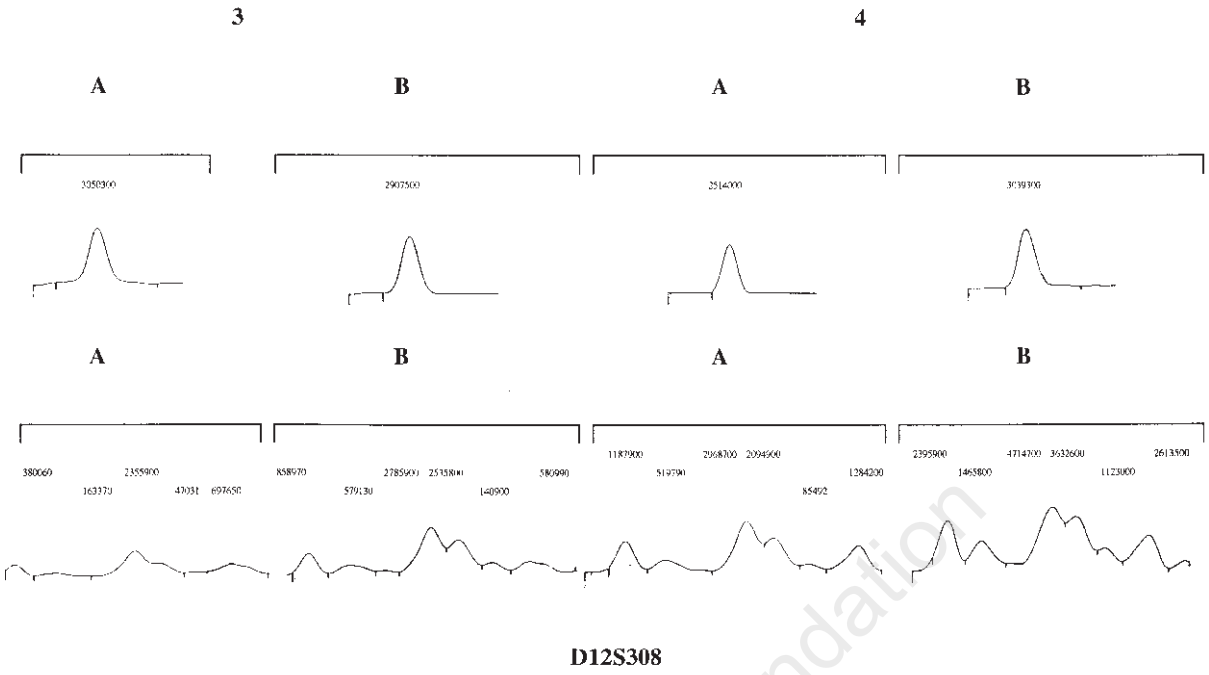

Figure 4. Densitometric analysis of radiographic signal intensity visible in the patterns of DNA extracted from bone marrow samples at remission compared with matched DNA at diagnosis in cases #3 and #4. The control of quality of the DNA loaded for each sample analyzed is represented at the top of the figure. The areas below the curve, which are measured by a densitometer between point to point represent the signal intensity of the PCR products analyzed.

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gested by Sato *et al.*,[26] making the KIP1 gene an excellent candidate suppressor gene. The DCC gene is another suppressor gene frequently deleted in colorectal cancer[27] and probably inactive in hematological malignancies.[28] This gene is expressed in normal bone marrow and peripheral blood lymphocytes, whilst being altered in acute leukemia and CML.[29] We have examined the possible implication of the DCC gene in childhood ALL using an informative microsatellite marker on chromosome 18q. The results obtained showed 1 case simultaneously altered with D2S123. We postulated a genetic damage which, in association with other abnormalities, is indicative of a debilitated interaction between hemopoietic cells and bone marrow microenvironment during the normal process of cell growth and differentiation.

In summary, we have examined the expression of microsatellite patterns in childhood ALL and have found that in a proportion of cases, of both B- and T-lineage, these patterns may be altered. This suggests that in the leukemogenic process, changes of microsatellite sequences are the result of nonspecific genetic alterations, such as errors of replication or repairs of DNA, which at the time of remission are corrected. Genomic instability is, therefore, a possible predisposing step which, in combination with other molecular alterations, might be linked to leukemic transformation. The evidence that therapy related secondary leukemias have a very high incidence of MI[30] further supports the predisposing role exerted by a mutator phenotype. The relative low frequency of instability in primary hematological malignancies reported in the literature[12,25,31,32] and in the present study is probably contributed by the small number of genomic sites screened and by the selection of markers known to be frequently involved in different solid tumors. We have analyzed the chromosomal localization of two putative suppressor genes, KIP1 on chromosome 12p and DCC on chromosome 18q, which could be linked with cytogenetic changes leading to leukemic transformation in ALL patients.[10,28] Using informative microsatellite markers, such as D12S308 and D18S58 flanking the KPI1 and DCC suppressor genes, we detailed MI as LOH which was not evident by cytogenetic analysis. It is possible that if more loci were examined, the percentage of positive cases would increase further.

Contributions and Acknowledgments

GR, GB, AC, AG and RF formulated the design of the study. GB and MCP provided the childhood acute lymphoblastic leukemia samples and in many cases extracted the genomic DNA. GR carried out the microsatellite analyses. RF was responsible for the funding of the study. All the authors contributed to the critical evaluation of the paper. GR, GB and RF were responsible of the writing of the paper. The order in which the names appear is a consequence of the above description. GR is the first author since she contributed

to the design of the study, carried out most of the experimental work and helped in writing the paper, while RF, the senior author, is the head of the group that performed the work, made contacts with the clinicians at the Dept. of Pediatrics in Padua, provided the funds and supervised the writing of the paper.

Funding

This work was supported by Istituto Superiore di Sanità, Rome, by the Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Rome, and by the Associazione Italian per la Ricerca sul Cancro (AIRC), Milan, Italy. GR was in receipt of a fellowship from Istituto Superiore di Sanità, Rome.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received June 19, 1997; accepted February 4, 1998.

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