

Analysis of loss of heterozygosity and X chromosome inactivation in spleens with myeloproliferative disorders and acute myeloid leukemia

Dennis P O'Malley, Attilio Orazi, Mingsheng Wang and Liang Cheng

Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, USA

Neoplastic myeloid proliferations are seen in the spleens of some patients with acute and chronic myeloproliferative disorders. Both acute myeloid leukemia (AML) and chronic myeloproliferative disorders have a variety of underlying cytogenetic defects that can be evaluated by loss of heterozygosity (LOH) studies. LOH studies have advantages over conventional cytogenetics by allowing the use of archival tissues. We evaluated the spleens in AML and chronic myeloproliferative disorders with neoplastic myeloid proliferations for the presence of LOH at several chromosome loci, and X-chromosome inactivation. A total of 17 spleens were evaluated (chronic myelogenous leukemia = 6; chronic idiopathic myelofibrosis = 6; essential thrombocythemia = 1; AML arising from previous chronic myeloproliferative disorders = 4). We examined LOH loci 7q (D7S2554), 8q (D8S263), 9p (D9S157, D9S161), 13q (D13S319), common sites of genetic abnormality in chronic myeloproliferative disorders, and *TP53*. In six cases, spleen LOH findings were compared to those of concurrent or preceding bone marrow biopsies. Five spleens of female patients were evaluated for the presence of clonality using X-chromosome inactivation. Of the 16 cases analyzed, 14 (88%) had at least one abnormal LOH locus, with 6/16 with two abnormal loci. The abnormalities were distributed as follows: D9S161–7/15 (47%), TP53–6/16 (38%), D7S2554–5/16 (31%), D9S157–5/15 (33%), D8S263–3/14 (21%), and D13S319–2/14 (14%). Of the six bone marrows, 4/6 showed concordance in bone marrow and spleen specimens, with additional LOH abnormalities being identified in the spleen specimens of all four cases. X-chromosome inactivation studies were showed nonrandom (clonal) patterns in two cases. Our results show that allelic losses were common in the neoplastic extramedullary hematopoiesis found in spleens of chronic myeloproliferative disorders and AML. Comparison of spleen and bone marrow specimens by LOH demonstrated additional abnormalities in the spleen compared to the marrow.

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Neoplastic hematopoietic proliferations can be seen in the spleens of patients with acute and chronic myeloid neoplasms. It occurs particularly frequently in patients with chronic myeloproliferative disorders, especially in chronic idiopathic myelofibrosis and chronic myelogenous leukemia (CML).^{1–3} In addition, spleen is a common site of

blastic transformation in chronic myeloproliferative disorder.⁴

Both acute myeloid leukemias (AML) and chronic myeloproliferative disorders have a variety of underlying molecular and cytogenetic defects. Loss of heterozygosity (LOH) has been used previously to detect genetic imbalances in these disorders, with several genetic loci being commonly involved. LOH evaluation provides an advantage over conventional cytogenetics because of the ability to evaluate archival, paraffin-fixed tissue samples. We chose to evaluate six chromosomal loci that have been shown to be frequently abnormal in chronic myeloproliferative disorder.^{5–8} We evaluated spleens with chronic myeloproliferative disorders and AML that

Correspondence: Dr DP O'Malley, MD, Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, 702 Barnhill Drive, Riley 0969, Indianapolis, IN 46202, USA.

E-mail: dpomalle@iupui.edu

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arose from previous chronic myeloproliferative disorder and with extensive neoplastic myeloid proliferations for the presence LOH at the selected loci. Further, we evaluated clonality in female patient samples using X-chromosome inactivation analysis. Finally, we evaluated LOH loci in six bone marrows with chronic myeloproliferative disorder that preceded or were concurrent with splenectomy specimens, and compared those results to the findings in the corresponding splenectomy.

Methods

Tissue Samples and Microdissection

Archival surgical materials from 17 patients with splenectomy were retrieved from the surgical pathology files of the Department of Pathology and Laboratory Medicine of the Indiana University School of Medicine (Indianapolis, IN). Cases were selected for the presence of extensive neoplastic myeloid proliferations (CML = 6; chronic idiopathic myelofibrosis = 6; essential thrombocythemia = 1; AML arising from previous chronic myeloproliferative disorder = 4). In six cases, bone marrow biopsies that preceded or were concurrent with the splenectomy specimens were retrieved and reviewed. Three cases with extensive benign EMH were included for comparison (autoimmune hemolytic anemia = 1, thrombotic thrombocytopenic purpura = 1, fibrocongestive splenomegaly = 1).

Histologic sections were prepared from formalin-fixed, paraffin-embedded tissue and were stained with hematoxylin and eosin for microscopic evaluation. Laser-assisted microdissection was performed (Figure 1), separating neoplastic myeloid proliferations from normal control tissues (residual lymphoid tissues) on unstained sections using a PixCell II Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA, USA), as previously described.⁹⁻¹⁴

Detection of LOH

The dissected cells were deparaffinized with xylene and ethyl alcohol. Polymerase chain reaction (PCR) was used to amplify genomic DNA at six specific loci on five different chromosomes: 7q31.1 (D7S2554), 8q24 (D8S263), 9p21 (D9S157, D9S161), 13q14 (D13S319), and 17p13 (TP53). Previous studies have demonstrated that LOH at these loci occurs in chronic myeloproliferative disorders.⁵⁻⁸ A summary of these loci, and their relation to important genes is presented in Table 1. PCR amplification and gel electrophoresis were performed as previously described.⁹⁻¹⁴ The criterion for allelic loss was complete or nearly complete absence of one allele in tumor DNA.⁹⁻¹⁴ PCRs for each polymorphic microsatellite marker were repeated at least twice from the same DNA preparations and the same results were obtained.

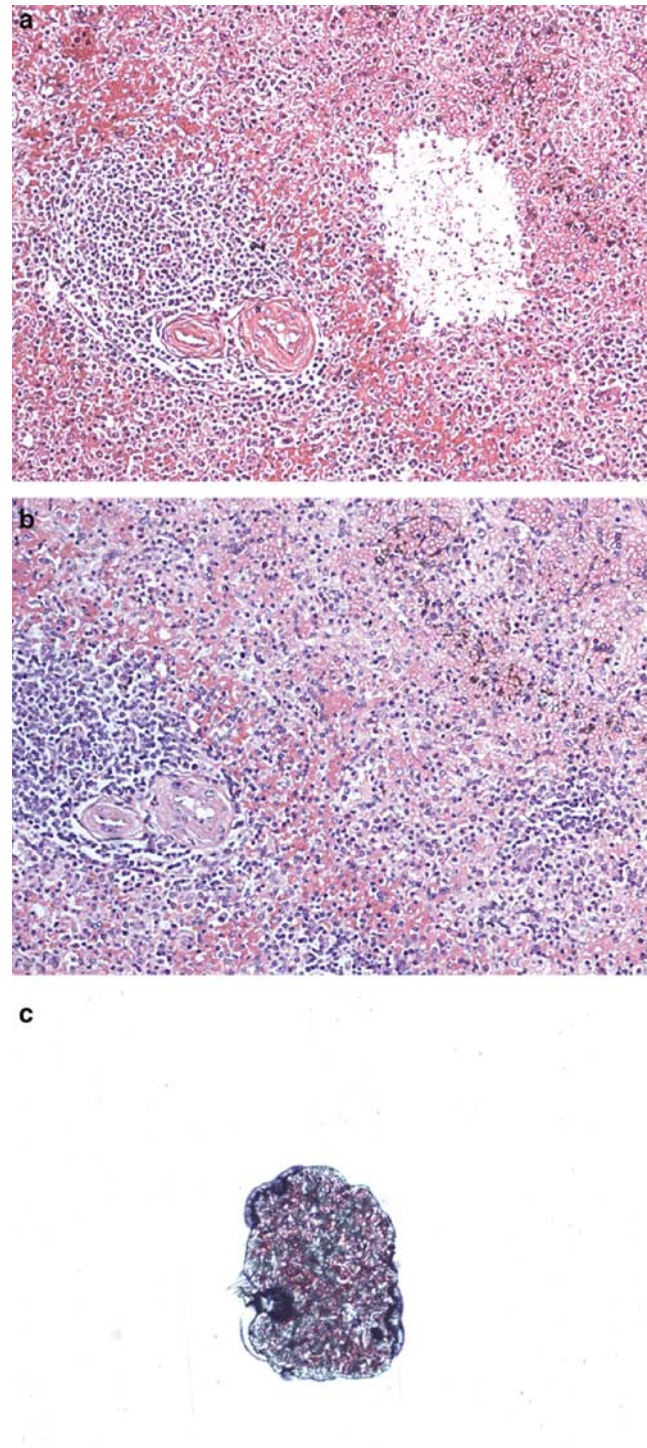


Figure 1 Laser capture microdissection. Splenic tissue with laser capture microdissection of red pulp (a), and same tissue before microdissection (b). (c) Tissue dissected, which was used for LOH or X-chromosome inactivation studies.

Detection of X-Chromosome Inactivation

X-chromosome inactivation analysis was performed on spleen samples from five female patients, using techniques previously described.¹¹ DNA samples were prepared as for LOH studies. The dissected

Table 1 LOH loci evaluated

Chromosome	Locus	Gene/location
7q31.1	D7S2554	Putative tumor suppressor gene
8q24	D8S263	Telomeric to <i>MYC</i>
9p21	D9S157	Telomeric to p16
9p21	D9S161	Centromeric to p16
13q14	D13S319	Telomeric to <i>RB</i>
17p13	p53	TP53

cells were placed in 50 μ l of buffer (ie 10 mM Tris-HCl, 1 mM EDTA, 1% Tween 20, and 3 mg/ml of proteinase K (pH 8.3) and incubated overnight at 37°C. The solution was boiled for 10 min to inactivate the proteinase K and used directly for subsequent clonal analysis without further purification. Aliquots (8 μ l) of the DNA extract were digested overnight at 37°C with 1 U of *HhaI* restriction endonuclease (New England Biolabs Inc., Beverly, MA, USA) in a total volume of 10 μ l. Equivalent aliquots of the DNA extracts were also incubated in the digestion buffer without *HhaI* endonuclease as control reactions for each sample. After the incubation, 3 μ l of digested or nondigested DNA was amplified in a 25- μ l PCR volume containing 0.1 μ l 32 [P] α -labeled deoxyadenosine triphosphate (dATP) (3000 Ci/mmol), 0.8 μ M AR-sense primer (5'TCC AGA ATC TGT TCC AGA GCG TGC3'), 0.8 μ M AR-antisense primer (5'GCT GTG AAG GTT GCT GTT CCT CAT3'), 4% dimethyl sulfoxide, 2.5 mM MgCl₂, 150 μ M deoxycytidine triphosphate, 150 μ M deoxythymidine triphosphate, 150 μ M deoxyguanosine triphosphate, 120 μ M deoxyadenosine triphosphate, and 0.13 U *Taq* DNA polymerase (Perkin-Elmer Corp., Norwalk, CT, USA). Each PCR amplification had an initial denaturation step of 95°C for 8 min, followed by 38 cycles at 95°C for 40 s, at 63°C for 40 s, and at 72°C for 60 s and then followed by a single final extension step at 72°C for 10 min. The PCR products were then diluted with 4 μ l of loading buffer containing 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanole FF (Sigma Chemical Co., St Louis, MO, USA). The samples were heated to 95°C for 5 min and then placed on ice. In total, 3 μ l of the reaction mixture was loaded onto 6.5% polyacrylamide-denaturing gels without formamide, and the PCR products were separated by electrophoresis at 80 W for 2 h. The bands were visualized after autoradiography with Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY, USA) for 8–16 h (Figure 2).

Analysis of X-Chromosome Inactivation

The cases were considered to be informative if two androgen receptor allelic bands were detected after

PCR amplification in normal control samples that had not been treated with *HhaI*. Only informative cases (ie, those without a skewed pattern of X-chromosome inactivation after being treated with *HhaI* in normal control samples) were included in the analysis. In tumor samples, nonrandom X-chromosome inactivation was defined as a complete or a nearly complete absence of an androgen receptor allele after *HhaI* digestion, which indicated a predominance of one allele.

Results

LOH Spleen

Of the 17 spleens with neoplastic myeloid proliferations studied, 16/17 had informative results in both normal and tumoral tissue for at least four of the six probes tested. These results are summarized in Table 2 and Figures 3–5. Of the 16 cases, 14 (88%) had at least one abnormal LOH locus, with 6/16 with two abnormal loci and 4/16 with three abnormal loci. The abnormalities were distributed as follows: D9S161–7/15, TP53–6/16, D7S2554–5/16, D9S157–5/15, D8S263–3/14, and D13S319–2/14. No LOH was seen in the paired negative control tissues. In the three spleens with benign EMH, no LOH was seen at any loci.

X-Chromosome Inactivation Studies

Of the five cases evaluated, one was noninformative. Of the remaining four cases, two had evidence of clonality as measured by nonrandom X-chromosome inactivation. Although clonal origin was not proven for two of the cases, these cases did show evidence of LOH abnormalities, suggesting clonality.

LOH: Comparison of Spleen and Bone Marrow

In six cases, bone marrow biopsies that preceded or were concurrent with the splenic biopsies were evaluated for the same panel of LOH loci abnormalities. In 6/6 cases studied, there were identified differences in the LOH patterns between the spleen and bone marrow samples. These results are summarized in Table 3. In general, 4/6 cases had one ($n = 3$) or two ($n = 1$) additional abnormalities in the spleen compared to the bone marrow. In one of the other cases (case 1—CML), the spleen showed no LOH abnormalities, while the marrow showed two abnormal loci. In the final case (case 6—chronic idiopathic myelofibrosis), there were two entirely

Figure 2 Examples of LOH and X-chromosome inactivation studies. Composite of LOH study results from several specimens. Each image (a–g) shows a normal, heterozygous result (left) and an abnormal, homozygous result (right) for (a) D7S2554, (b) D8S263, (c) D9S157, (d) D9S161, (e) D13S319, (f) TP53, and (g) X-chromosome inactivation.

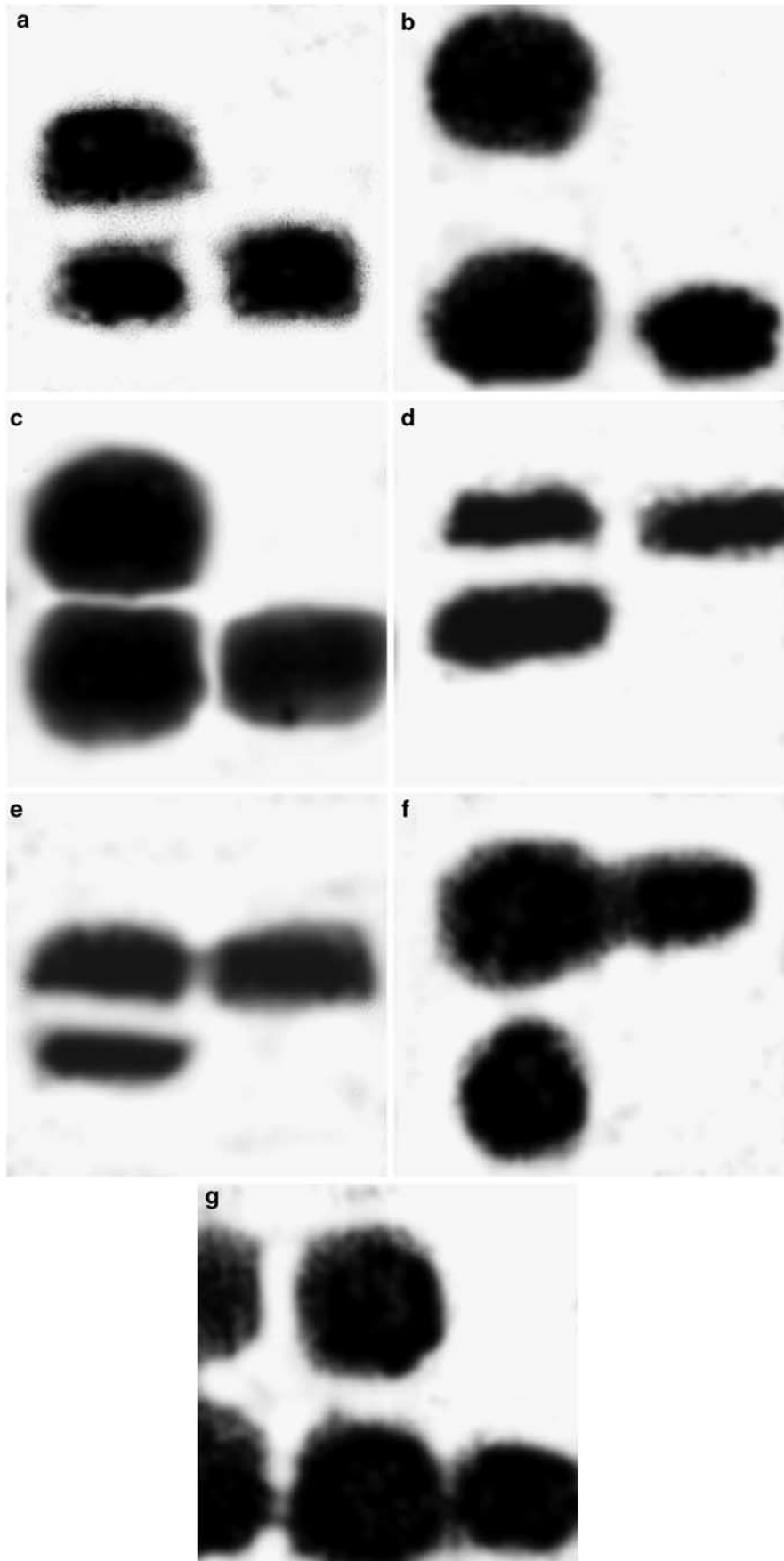


Table 2 Results of LOH and X chromosome inactivation studies

Case	Dx		D7S2554	D8S263	D9S157	D9S161	D13S319	TP53	X chr
1	CIMF	C	0	0	0	0	0	0	
		T	+	+	+	0	0	0	
2	CIMF	C	0	0	0	0	0	0	
		T	0	0	0	+	0	+	
3	CIMF	C	0	0	0	0	0	0	
		T	+	0	0	0	0	+	
4	CIMF	C	0	0	0	0	0	0	N
		T	0	0	0	0	0	0	N
5	CIMF	C	0	N	0	0	0	0	0
		T	0	N	+	0	0	0	+
6	CIMF	C	0	0	0	0	0	0	0
		T	0	0	+	+	0	0	0
7	CML	C	0	0	N	0	0	0	
		T	+	0	N	+	0	+	
8	CML	C	0	0	0	0	0	0	
		T	0	0	0	0	0	+	
9	CML	C	0	0	0	0	N	0	
		T	0	+	0	+	N	+	
10	CML	C	0	N	0	N	0	0	
		T	0	N	0	N	+	+	
11	CML	C	0	0	0	0	0	0	
		T	0	0	0	0	0	0	
12	CML	C	0	0	0	0	0	0	
		T	0	0	0	+	+	0	
13	AML	C	N	N	N	N	N	N	
		T	N	N	N	N	N	N	
14	AML	C	0	N	0	0	N	0	
		T	+	N	+	+	N	0	
15	AML	C	0	0	0	0	0	0	
		T	0	+	0	0	+	0	
16	AML	C	0	0	0	0	0	0	0
		T	+	0	+	0	0	0	+
17	ET	C	0	0	0	0	0	0	0
		T	0	0	0	+	0	0	0

C=control tissue; T=neoplastic splenic tissue; 0=normal result; +=allelic loss; N=noninformative study; AML=acute myeloid leukemia; CML=chronic myelogenous leukemia; CIMF=chronic idiopathic myelofibrosis; ET=essential thrombocythemia; X chr=X-chromosome inactivation analysis.

different, and mutually exclusive, sets of abnormalities found in the two locations.

Discussion

Our results show that LOH abnormalities are common in the neoplastic EMH found in spleens of chronic myeloproliferative disorders and AML.

We found LOH abnormalities common to myeloproliferative disorders in most of the studied spleens (88%). One to three LOH abnormalities were seen in 5/6 chronic idiopathic myelofibrosis, 5/6 CML, 1/1 essential thrombocythemia, and 3/3 AML. The fact that such a high percentage of cases showed abnormalities by LOH analysis suggests that this methodology might be used as an adjunct to routine histopathologic diagnosis of these types of disorders

in the spleen when conventional cytogenetics are not available. Further, in many cases, multiple abnormalities were seen.

Evidence of nonrandom X chromosome inactivation was seen in 2/4 of informative cases analyzed, confirming the clonal nature of the process. However, in the other two cases, nonrandom X chromosome inactivation was seen. Several studies have shown that random X chromosome inactivation may be observed in up to 50% of invasive cancers.¹¹ There are a number of possible explanations including (1) incomplete digestion of DNA samples prepared from formalin-fixed, paraffin-embedded tissues, (2) contamination by normal tissues, (3) the presence of X chromosome aneuploidy, (4) the coexistence of multiple tumor subclones of independent origins, (5) reactivation of inactive X chromosome-linked genes, and (6) variable methylation patterns at the CpG sites of the androgen receptor locus. Rarely, other mechanisms may attribute to the inactive status of X chromosome in female subjects.¹¹

The results of the follow-up studies, comparing previously or concurrently obtained bone marrow biopsies to spleen samples, provided some interest-

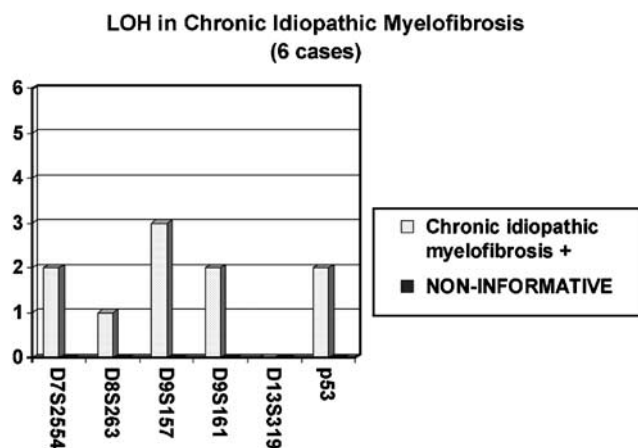


Figure 3 LOH abnormalities in chronic idiopathic myelofibrosis. Summary of LOH findings in six chronic idiopathic myelofibrosis cases.

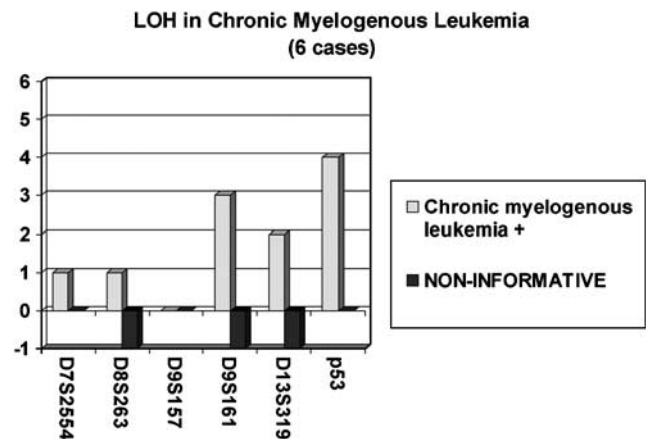


Figure 4 LOH abnormalities in CML. Summary of LOH findings in six CML cases.

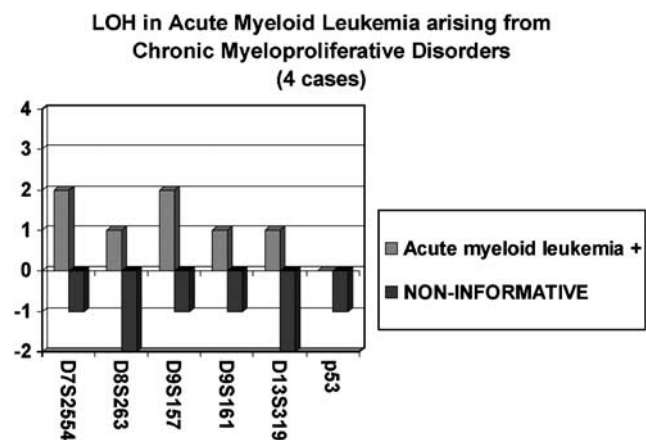


Figure 5 LOH abnormalities in AML arising from previous chronic myeloproliferative disorders. Summary of LOH findings in four AML cases.

Table 3 Summary of findings in six cases comparing bone marrow and splenic LOH results

	Site	D7S2554	D8S263	D9S157	D9S161	D13S319	p53
Case 2, CIMF	Spleen	0	0	0	+	0	+
CIMF	Marrow	+	+	0	0	+	0
Case 10, CML	Spleen	0	N	0	N	+	+
CML	Marrow	0	N	0	N	0	+
Case 11, CML	Spleen	0	0	0	0	0	0
CML	Marrow	0	+	0	0	0	+
Case 12, CML	Spleen	+	0	N	+	0	+
CML	Marrow	0	0	N	+	0	0
Case 14, AML	Spleen	+	N	+	+	N	0
MPD, U	Marrow	+	N	0	+	N	+
Case 15, AML	Spleen	0	+	0	0	+	0
CMML	Marrow	0	0	0	0	+	0

'+' indicates allelic loss; red '+' indicates discordance in either marrow or spleen findings. '0' indicates normal finding. 'N' indicates a noninformative finding at an individual locus. AML=acute myeloid leukemia; CIMF=chronic idiopathic myelofibrosis; CML=chronic myelogenous leukemia; CMML=chronic myelomonocytic leukemia; MPD, U=chronic myeloproliferative disease, unclassifiable.

ing results. In four of the six cases, the spleen showed additional abnormal LOH loci compared to preceding marrows. This supports the observation that the spleen is a common site of clonal evolution and possibly blast transformation.¹⁵ In the single case of CML where the spleen showed no abnormal loci and the marrow did have LOH abnormalities, there are at least two possibilities that may account for this: (1) the spleens were a site of non-neoplastic hematopoiesis compared to abnormal marrows or (2) the spleens did not have any of the LOH abnormalities tested while the marrow had clonal progression compared to the spleen. It is difficult to distinguish which of these possibilities is more likely. The single case of chronic idiopathic myelofibrosis studied showed the most surprising results. The presence of multiple different and mutually exclusive LOH abnormalities between bone marrow and spleen may suggest that chronic idiopathic myelofibrosis is more clonally unstable with more heterogeneous lineage involvement than other chronic myeloproliferative disorder.⁶

In a recent study by Mesa *et al*¹⁵ using conventional cytogenetics, bone marrow and splenic karyotypes were compared in chronic idiopathic myelofibrosis. In this study, it was found that additional karyotypic abnormalities found in the spleen were associated with significantly worse postsplenectomy survival.

In conclusion, we demonstrate that clonal abnormalities are frequently present in spleens with neoplastic myeloid proliferations using LOH and X-chromosome inactivation analysis. The nature and evolution of neoplastic myeloid proliferations in the spleen, particularly in chronic myeloproliferative disorder, is a potentially rich area of future research.¹⁶ Although it is unlikely to be used as a primary diagnostic method, LOH studies may be of benefit in understanding nature of evolution of clonal cells and the detection of abnormal genetic findings in chronic myeloproliferative disorder and other hematopoietic disorders.

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