Deletion Mapping on the Long Arm of Chromosome 7 in Splenic Lymphoma With Villous Lymphocytes

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Splenic lymphoma with villous lymphocytes (SLVL) is a low-grade lymphoproliferative disorder characterized by splenomegaly and circulating villous lymphocytes in the peripheral blood. It is considered to be the leukemic form of splenic marginal zone lymphoma (SMZL). The genetic basis of this lymphoma type remains unknown. Conventional cytogenetic studies have identified frequent structural abnormalities of chromosome 7, in the form of translocations, mainly unbalanced, and 7q deletions. In this current study, we undertook deletion mapping of the long arm of chromosome 7 in a series of cases with SLVL. Metaphase fluorescence in situ hybridization (FISH) was used in the first instance, followed by a study of loss of heterozygosity (LOH). The common area of deletion identified by FISH spanned from the YAC clone HSC7E1289 (mapping to 7q32.1) to in between YACs HSC7E195 and HSC7E648 (7q32–3). By application of 50 microsatellite markers mapping to the FISH-CDR and to areas of deletion reported in other studies, four distinct hotspot loci were identified, with abnormalities present in 29–55% cases. In three of them, both LOH and biallelic deletions were found. The LOH in the majority of patients was noncontiguous. The presence of a high incidence of abnormalities in the established hotspot areas and in particular the finding of biallelic deletions is indicative of the existence of genes important for the pathogenesis of SLVL in these areas.

INTRODUCTION

Abnormalities of the long arm of chromosome 7 are the most common karyotypic changes in splenic lymphoma with villous lymphocytes (SLVL) (Oscier et al., 1993), a low grade B-lymphoproliferative disorder characterized by the presence of splenomegaly and circulating villous lymphoid cells in peripheral blood (Catovsky and Matutes, 1999). However, there is no single recurrent aberration, and alterations include both deletions and translocations to several different chromosomes (Oscier et al., 1993, 1996). Most of the translocations described are unbalanced and therefore result in the loss of chromosomal material from the long arm of chromosome 7. In view of the occurrence of 7q deletions and unbalanced translocations in SLVL, we undertook deletion mapping on the long arm of chromosome 7, with the aim of delineating the minimal common deleted region(s) (CDR), which may potentially harbor a gene(s) relevant to the pathogenesis of SLVL. Mapping by fluorescence in situ hybridization (FISH) with 46 yeast artificial chromosome (YAC) clones was adopted in the first instance on a series of 13 SLVL patients with known abnormalities of chromosome 7. This was followed by mapping by loss of heterozygosity (LOH) through the use of 50 microsatellite repeat markers from within the FISH-CDR and covering the regions reported previously to be deleted in patients with splenic lymphomas (Hernandez et al., 1997b; Mateo et al., 1999). This latter study was performed to search for microdeletions on a series of 29 patients with apparently normal or unknown cytogenetic 7q status.

MATERIALS AND METHODS

Patients

The diagnosis of SLVL was established on the basis of peripheral blood morphology (Matutes et al., 1994a), immunophenotype (Matutes et al., 1994b), clinical features (Matutes et al., 1994a; Catovsky and Matutes, 1999), and, when available, spleen and/or marrow histology (Isaacson et al., 1994). A group of 13 patients with known abnormalities of the long arm of chromosome 7 were tested in the FISH part of this study. The karyo-

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TABLE 1. Karyotypes of Patients With 7q Abnormalities Studied in the FISH Part of This Project

No.	Case ID	Karyotype	Source[a]
1	MZL1	46,XX,dup(1)(q12q32) add(1)(?p36), t(2;9;14)(p12;p13;q32), del(7)(q22q33), der(17)t(12;17)(q21;p12)	(Morrison, et al., 1998)
2	BET	46,XX,der(7)t(3;7)(q13.3;q21)	(Troussard, et al., 1998)
3	HK	46,XX,del(7)(q22q32),inv(10)(p13q23)[12]/46,XX[7]	(Wong, et al., 1998)
4	961827	47,XY,+add(7)(q32)	Dr. C. B.
5	971251	46,XX,add(7)(q21)	Dr. C. B.
6	G522	46,XY,?add(1)(q43),der(7)t(3;7)(q21;q31),?add(14)(q32)[18]/46,XY[4]	Dr. E. C-B.
7	M483	46,XX, der(7)t(3;7)(q?;q34)[15]/46,XX[10]	Dr. E. C-B.
8	R017	51,XX,+X,add(1)(q43),+der(3)t(3;?)(p13;?),del(6)(q14–15;q26), del(7)(q31),+del(7)(q22),−8,−15[6],−17[3],+18,+18,−22[5],+1–6 mar/100–103,idem×4[3]/46,XX[3]	Dr. E. C-B.
9	R061	46,XY,t(6;10)(q15;q22),del(7)(q22)[11]/46,idem, der(6)add(6)(p25)del(6)(q15q23)[2]/46,XY[7]	Dr. E. C-B.
10	S498	46,XX,der(7)t(7;12)t(7;12)(q31;q12)[19],46,XX[1]	Dr E. C-B.
11	72/99	46,XX,der(7)t(7;12)(q22;q13),t(10;11)(q11;p?13)/46,XX	RMT
12	207/99	47–8,XX,del(3)(p13),del(3)(q13),+3,add(6)(q27),del(6)(q22),der(7)(X;7)(q2?2;q31),+mar1,+mar2[3]/46,XX[2]	RMT
13	110/01	46,XX,del(7)(q21:q31)	RMT

[a]Legend: Dr E. C-B, Dr. Evelyne Callet-Bauchu, Lyon, France; Dr C. B., Dr. Christian Bastard, Rouen, France; RMT, Royal Marsden NHS Trust.

types are included in Table 1. The LOH study was performed on 32 samples from 29 patients with apparently normal or unknown karyotype.

Metaphase FISH

Whole blood nonstimulated overnight and stimulated 3- and 5-day cultures were carried out through the use of the tumor-promoting agent (TPA) mitogen (phorbol 12-myristate 13-acetate; Sigma, Poole, UK) at the concentration of 20 mg/L. Before harvesting the cultures, mitotic spindle formation was inhibited by addition of Colcemid followed by incubation with hypotonic KCl (0.075 M) and fixation with Carnoy's fixative (3:1 methanol: acetic acid). Slides for FISH were made the day before they were required.

Detailed information on the clones used for FISH experiments can be viewed on the Chromosome 7 Project website (http://www.genet.sickkids.on.ca/chr7db/) and is summarized in Table 2. The initial set of probes was chosen to provide coverage of one probe per cytogenetic band for approximate localization of where the breakpoints of translocations and deletions were. Identification of a breakpoint in between two probes was further investigated by FISH by use of probes mapping between the first set. Thus, 46 clones total were used in this study. Clones were grown and their total genomic DNA was extracted by use of standard methods. DNA was labeled by nick translation (Biotin- and DIG-Nick Translation kits from Roche Diagnostics, Lewes, UK) as indicated by the manufacturer. The hybridization was conducted as previously described (Gruszka-Westwood et al., 2001). A digoxigenin-labeled centromeric probe for chromosome 7 (Qbiogene, Edinburgh, UK) was combined with a YAC of interest and a 7q35–6 cosmid for easier identification of the homologs of 7 (marking with centromeric 7 probe) and of the abnormal one (cosmid missing or closer to the centromere). In cases with few metaphases, only the centromeric probe was used with a YAC of interest, to exclude the possibility of the presence of monosomy 7.

LOH Analysis

DNA source

Tumor DNA was extracted from peripheral blood and/or spleen tissues by separating low density cells over Histopaque (<1.077 g/ml; Sigma) and removing monocytes and T cells. To eliminate the monocytes, samples were incubated with L-leucine methyl ester, as adapted from Thiele et al. (1983); the T cells were removed by the sheep red blood cell rosetting method. This procedure resulted in achieving purity of over 90% for the B-cell samples. High molecular weight DNA was extracted according to standard methods. Germline DNA was obtained from the epithelial cells contained in the patient's mouthwash samples and was extracted by boiling the pelleted cells for 20 min in 200 μl of 0.05 M NaOH. The solution was then neutralized with 20 μl of 1 M Tris–HCl, pH 7.7. After pelleting of proteins and cellular debris, the supernatant containing DNA was used for PCR.

TABLE 2. Details on YAC Probes Used for the Mapping by FISH on 7q in SLVL

Clone name	Localization	Size	Loci
CEPH 845h11	7q11.23–q21.1	1600	*semaphorin E* (sts-*N38844*), *CD36, D7S660, D7S2791, D7S634, D7S1797, sWSS4014, sWSS2121, sWSS4015*
CEPH 877e7	7q21.1–q21.2	1690	*D7S166, D7S195, D7S644, D7S2481, GRM3, D7S2555, D7S2343, AFMB320VH1*
HSC7E590	7q21.1–q21.2	460	*D7S166, D7S195, D7S260, D7S524, D7S644, D7S2537*
HSC7E962	7q21	500	*D7S644, GRM3, D7S2481*
cos87c3 (cos477)	7q21.1–q21.2	na	*GRM8*
HSC7E313	7q21.1	540	*D7S175, D7S236, D7S244, D7S580, D7S736, D7S2555*
HSC7E465	7q21.3	700	*CALCR, D7S15, D7S657, D7S652, D7S2430*
HSC7E1214	7q21.3–q22.1	980	*ASNS, TAC2, D7S82, D7S554, UT901, pG162, 932D8.2, D7S2370*
CEPH 747f2	7q22	1160	*D7S1813, D7S2313, D7S1503, sWSS2669, sWSS3320, sWSS2144, sWSS2090, D7S1604, AFM290YG9, D7S2509, PSMC2, D7S1663, RELN*
CEPH 887d10	7q22	100	*D7S515*
CEPH 964h9	7q22	1350	*ORC5L, D7S1799, D7S658, D7S796, D7S818, AFM211ZB8, D7S2446, AFMB296YE5, CHLC.GATA4E02, CHLC.GATA6G06, D7S2504, D7S1604, AFM290YG9, D7S2509, PSMC2, D7S1663, RELN, sWSS1679, sWSS2200, D7S2494, sWSS2968, sWSS638*
HSC7E56	7q22	460	*SRPK2, D7S576, HSC7E56LV, D7S1530*
HSC7E873	7q22–q31.1	440	*PBEF, D7S2453, NIB349, D7S2305*
HSC7E957	7q22	360	*PBEF*
HSC7E179	7q22.3–q31.1	560	*D7S216, sWSS1607, sWSS1619 D7S499, D7S659, CHLC.GATA3B01, D7S2357, D7S2550, AFM330YG5, 60plc2, 61pla12, 61plcl, 61plc2, 61plfl, 61plf3, 61plg2, 62plel2,*
CEPH 927a1	7q22	960	*D7S2783, D7S2780, D7S2773, D7S496, D7S2333, D7S1412, PRKAR2A, sWSS2841, D7S2420, D7S1409, D7S1919, sWSS699, D7S1556, sWSS1826, D7S496, sWSS2394, D7S2317*
cos5h2 (cos732)	7q31.2	na	*M6 (near MET)*
HSC7E813	7q31.2–q31.3	900	*CAV1, CAV2, D7S522, D7S567, D7S568*
HSC7E127	7q31.2–q31.3	560	*CFTR, WNT2, D7S633, D7S677*
HSC7E146	7q31.3–q32	1000	*D7S8, D7S193, D7S655*
HSC7E39	7q32–q33	na	*D7S2487, D7S2039E (Cda0UH06)*
HSC7E582	7q31.3	510	*D7S643*
cos81g2 (cos375)	7q32	35	*GRM8, E1326cd666*
HSC7E256	7q31.3–q33	600	*D7S480, D7S650, D7S685, D7S2158E, (Cdy0rd04), D7S2821*
HSC7E619	7q33–q34	380	*ZP3 (sWSS1286), D7S640, D7S538E, AFM287VC9, D7S1845*
HSC7E448	7q31.3–q33	580	*D7S490, D7S2529*
HSC7E451	7q31.3–q33	590	*SPAM1, HYAL4, HYALP1, D7S487, D7S648, D7S2520, cDNA 108*
HSC7E107	7q31.3–q33	580	*D7S87, D7S2471, D7S2225, D7S1835, GATA-P32047*
H_DJ0054P14	7q31.3–32.1	na	*GRM8*
HSC7E1289	7q31.3–32.1	1800	*PAX4, ARF5, GRM8, D7S686, D7S1801, D7S1822, D7S1874, D7S1873, D7S680, D7S514, D7S2244, D7S2378, D7S2779, D7S1498, WI-6035, HG8EB-1*
HSC7E125	7q32	560	*PODXL, D7S649, D7S1575, D7S2531, 164P22-9, 62D21-1.1, 62plf3, D7S1418, D7S2959*
HSC7E195	7q32	560	*MEST, CPA, D7S786, D7S2519, D7S2544, D7S1861, IB3560, C15A02*
HSC7E648	7q33–q34	na	*D7S681, pG528, D7S2215, D7S2437, D7S2452*
HSC7E1175	7q33–q35	1250	*ALDR1, BPGM, D7S500, D7S2553, D7S800, CALDI, Bdy91G08, 60p2b4, NOT4H*
HSC7E87	7q33	500	*PTN (HBNF), D7S2468, D7S2505*
CEPH 790h2	Na	1080	*63pld4, D7S2468, WI-8921, WI-10063, WI-4183, CHLC.GATA63F08*
HSC7E273	7q34–q35	450	*D7S545E*
HSC7E164	7q33–q34	500	*cd609, D7S810E, AFMB336XH9, SSBP, D7S2513, Bda73B01 D7S661, NIB1354, NEDD2, D7S2806, STSG-10096, WI-9353, D7S794, CHLC.GATA81D02, TCRB, PRSS1, sWSS3558, D7S1941, 7P05E05,*
CEPH 940a12	7q34–q35	1790	*sWSS2792, 7P05G09, KEL, D7S775, PIPI, 7P04E01, sWSS1032, CLCN1, sWSS2109, sWSS1686, sWSS1958, sWSS3100, sWSS2380, sWSS1691, D7S1613*
cos58c5 (cos737)	7q33–q35	na	*PIP*
CEPH 761h5	7q34–q35	750	*D7S498, D7S661, D7S676, D7S1803, NIB1354, D7S794, CHLC.GATA81D02, sWSS1958, sWSS3100, sWSS2380, sWSS1691, D7S1613, sWSS1791, sWSS2858, sWSS2140, D7S1573, D7S1665, sWSS1821, D7S1486, sWSS2869, TIMI, D7S1925, sWSS694, sWSS1849, sWSS1919, sWSS2155*
CEPH 846e8	7q34	1100	*D7S1836, D7S2511, AFMA082XC9*
CEPH 803g1	7q35	1080	*Hs-cul-1 (BCD3347), D7SG29, D7S688, HY5, sWSS2625, sWSS3922, D7S2419, D7S2442, HY4, HY3, D7S1510, sWSS2064, 7P05H10*
CEPH 647f12	7q35–q36	1390	*D7SG29, D7S688*
cos159g3 (cos595)	7q35–q36	na	*CDK5, D7SG31*

Selection of microsatellite markers

Fifty markers mapping to the FISH-CDR and also covering areas of deletion identified by previous studies were selected for use in the LOH study. Fine mapping and localization of the markers were obtained by use of Seqbase software (in-house software), which provided the physical distance between the markers and their order along chromosome 7, by lining up each marker to the contig containing it from the Human Genome Project physical maps at www.ensembl.org and www.ncbi.nih.nlm.gov by use of output from BLAST (www.ncbi.nih.nlm.gov). Physical distances between the ordered markers were calculated by assuming that each marker lies in the middle of its contig. The interval between the markers was calculated by subtracting the physical position of one marker from the position of the more telomeric one. The integrity of the map was checked by designing a primer for the DNA sequence upstream from the microsatellite forward primer and performing a PCR with the microsatellite reverse primer. The order and distance between the microsatellite markers are shown in Table 3.

PCR

PCR reactions were performed oil-free in 96-well microtiter plates at the final volume of 25 μl by use, per well, of 0.5 μl of genomic DNA (1 mg/ml), 2.5 μl 10× Thermoprime Plus *Taq* buffer, 1.5 μl $MgCl_2$ (25 mM), 1 μl dNTP (10 mM), 0.5 μl Thermoprime Plus *Taq* polymerase (1 U/μl) (all reagents from ABgene, Epsom, UK), 0.5 μl of each primer (5 μM), and water. The PCR cycle constituted 30 cycles of denaturation at 94°C for 30 sec, annealing at various temperatures for 75 sec, extension at 72°C for 15 sec, and a final extension step for 10 min. Primer sequences, predicted product size, allele number, and percentage heterozygosity were obtained from the Genethon website (www.genethon.fr), Genome Database website (www. gbd.org), and in some cases from the NCBI Entrez Nucleotide website (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=Nucleotide). The annealing temperatures were established through use of the OSP program available from the HGMP website (www.hgmp.mrc.ac.uk). Primers were obtained from ABI (Warrington, UK), and the forward primer was fluorescently labeled. The markers were divided into three sets based on their sizes, and the colors of the labels were assigned so that the markers with similar expected length were labeled differently.

Genescan analysis

The PCR products for the LOH study were analyzed on 4.5% polyacrylamide (29:1 acrylamide: bisacrylamide) denaturing gel in 1× TBE buffer (National Diagnostics, Manville, NJ) by use of an ABI 377 automated fluorescent DNA sequencer (ABI). Three microliters of each PCR reaction were combined with 3 μl blue dye with formamide and 1 μl of a ROX 500 fluorescent size marker (ABI). The size standard calling for each lane and the quality of data were checked automatically by use of the GSQC version 1.1 software (in-house software; ICR, Sutton). The results were then imported into Genotyper (version 2.5; ABI) for analysis. Each fluorescent peak was quantified in terms of size (in bp), peak height, and area under the peak.

Hotspot Analysis

The data were normalized by calculating the percentage LOH by the following equation: % Abnormal = (DEL + LOH)/(Total no. patients − UI) × 100, where DEL stands for a biallelic deletion and UI for uninformative cases homozygous for a given marker in the germline. Only regions for which >25% of cases were abnormal were considered to be hotspots.

RESULTS

FISH

By use of YAC and cosmid clones with defined physical location on the long arm of chromosome 7, a FISH-CDR was established for a series of patients with SLVL presenting with karyotypic changes involving 7q. Table 4 details the results of the FISH experiments in the informative cases, and Figure 1 shows the results in graphic form. Based on the results of mapping by FISH, the CDR was established to be located from the YAC probe HSC7E1289 (7q32) to in between HSC7E195 and YAC HSC7E648 mapping (7q33) (Table 4). The centromeric boundary of the CDR was established in patient 207/99 and the telomeric in patient 110/01. The proximal deletion breakpoints showed marked heterogeneity among the cases with 7/12 (58%) patients exhibiting breaks within the band 7q22 (BET, G522, 97-1251, R061, 72/99, R017, and HK), one case at 7q10 (96-1827), one on the boundary between 7q22 and 7q31 (M483), three within the band 7q31.2 (S498, R017,

TABLE 3. Order and Distance Between the Microsatellite Markers Used in the LOH Study

Marker	Contig (Ensembl map)	Interval bp	Interval Mb	Approximate cytogenetic position
D7S503	AC007091.00001	0	0.00	p21.1
D7S655	AL163247.00001	7917367	7.92	q21.3
D7S2502	AC034112.3.45365.59420	91671683	91.67	q31.2
D7S687	AC017003.00001	48145	0.05	q31.1
D7S486	AC073130.00001	298196	0.30	q31.2
D7S2097E	AC002542.1.1.188741	876988	0.88	q31.2
D7S633	AC002465.1.1.155881	243768	0.24	q31.2
D7S677	AC000111.00001	180575	0.18	q31.31
D7S2742	AC000061.1.1.82512	149354	0.15	q31.31
AFMA073ZB9	AC007874.00001	623199	0.62	q31.31
D7S480	AC006364.00001	2874051	2.87	q31.32
D7S2486	AC006009.2.1.93942	1232559	1.23	q31.33
D7S685	AC004875.00001	612061	0.61	q31.32
D7S490	AC073054.00001	1640936	1.64	q31.33
D7S487	AC006333.00001	443096	0.44	q31.33
D7S648	AC006148.00001	600160	0.60	q31.33
D7S686	AC003961.00001	1657844	1.66	q31.33
D7S504	AC006529.1.1.178069	157952	0.16	q31.33
D7S2501	AC006529.1.1.178069	102705	0.10	q31.33
D7S1873	AC005583.00001	607536	0.61	q32.1
D7S1801	AC002084.00001	563244	0.56	q32.1
D7S1822	AC002084.00001	50000	0.05	q32.1
D7S1874	AC002533.00001	174322	0.17	q32.1
D7S680	AC000361.00001	282819	0.28	q32.1
D7S514	AC000127.00001	355274	0.36	q32.1
D7S635	AC073934.00001	229138	0.23	q32.1
AFMC016YC9	AC006529.00001	236442	0.24	q32.1
W122238	AC018635.00001	157197	0.16	q32.1
D7S2072E	AC084864.1.100477.177660	144199	0.14	q32.2
D7S530	AC016695.00013	490597	0.49	q32.2
D7S2544	AC024085.5.1.168405	56042	0.06	q32.2
D7S2107E	AC007938.1.1.167237	168155	0.17	q32.2
D7S2531	AC009362.8.1.160156	833919	0.83	q32.3
D7S649	AC013434.00002	1464295	1.46	q32.3
D7S512	AC018643.00001	1103828	1.10	q32.3
D7S2452	AC007790.1.1.207791	228358	0.23	q33
D7S2437	AC009180.9.1.163384	85438	0.09	q33
D7S640	AC008038.00001	1053867	1.05	q32.3
D7S631	AC008154.00001	1444544	1.44	q33
D7S500	AC009331.00001	788243	0.79	q33
AFMA129WE9	AC009264.6.1.164824	204585	0.20	q33
D7S2735	AC018728.5.1.181933	232999	0.23	q33
D7S2560	AC008265.15.1.170537	1249085	1.25	q34
D7S2450	AC020983.6.60413.176594	179366	0.18	q34
D7S509	AC009179.00001	932153	0.93	q33
D7S495	Unmapped	Unmapped	Unknown	q34
D7S1824	AC091440.2.125303.139501	2271657	2.27	q34
D7S684	AC018663.00001	1268097	1.27	q34
D7S111	AC004889.1.1.130030	10960	0.01	q34
D7S2195	AC004928.2.1.137137	1466718	1.47	q34

and MZL1), and one on the 7q31.3–7q32 boundary (207/99). One case (96-1827), assigned by conventional cytogenetics as +add(7)(q32), showed no hybridization on the abnormal homolog with any of the clones used; however, because cells from this case had an extra copy of chromosome 7, there was no net loss of 7q sequences but gain of 7p. Thus, excluding the case 96-1827 with the breakpoint at the centromere of chromosome 7, the proximal breakpoints were scattered along a large genomic segment extending from sequences recognized by the YACs HSCE873 and HSC7E1289. The hybrid-

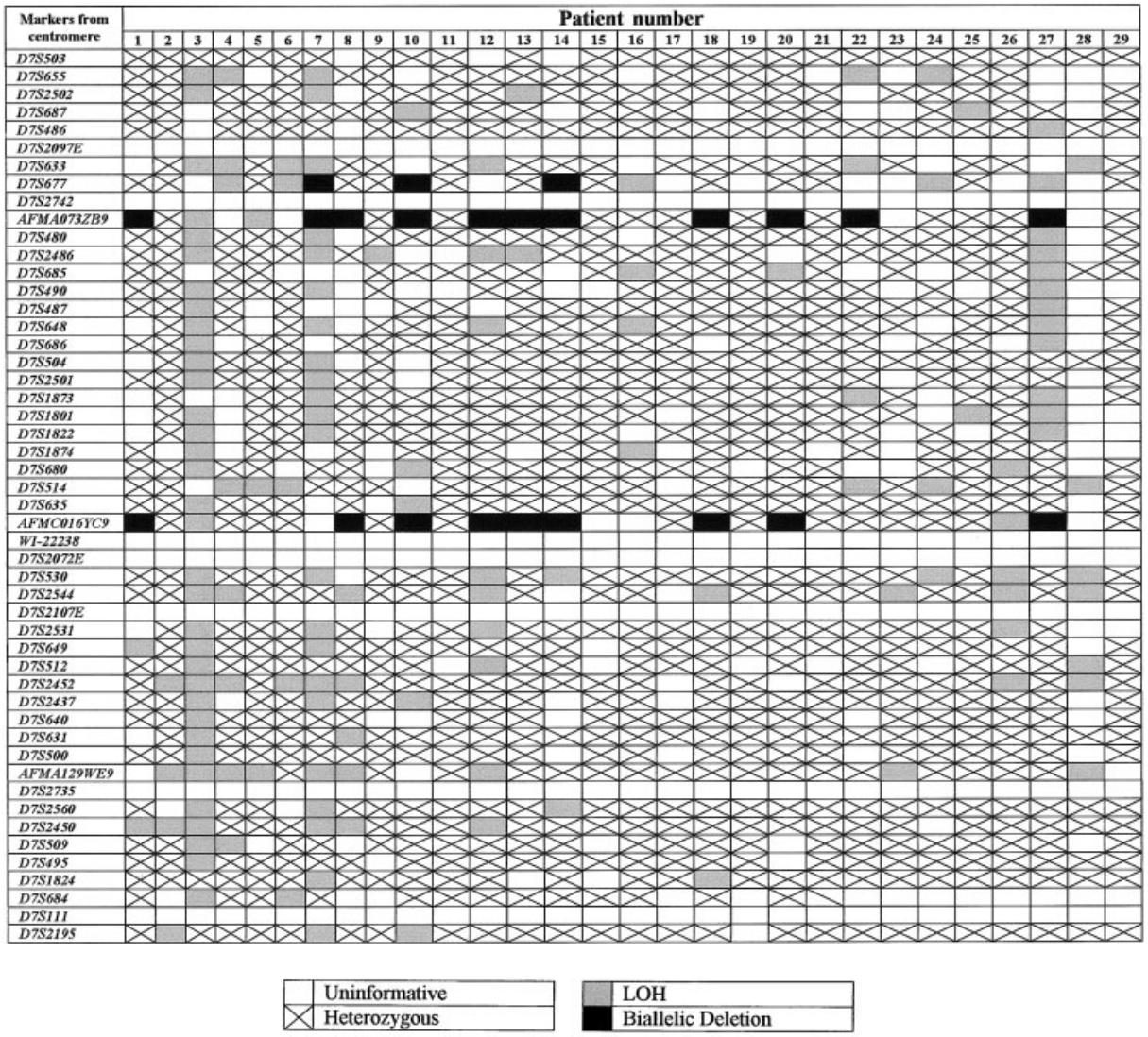
TABLE 4. FISH Results

Probes used	Location on 7	BET	G522	97-1251	RO61	72/99	S498	M483	RO17a*	RO17b	207/99	HK	MZL1	110/01
CEPH 845h11	7q21.1													
CEPH 877e7	7q21.1-22													
HSC7E590	7q21.1													
HSC7E962	7q21.1													
cos 477	7q21													
HSC7E313	7q21.1													
HSC7E465	7q21.2													
HSC7E1214	7q21.3													
CEPH 474f2	7q21.2-21.3													
CEPH 887d10	7q22													
CEPH 964h9	7q22													
HSCE56	7q22													
HSCE873	7q22													
HSCE957	7q22													
HSCE179	7q22													
CEPH 927a1	7q22													
cos 482	7q22.3-31													
cos 732	7q31.2													
HSC7E813	7q31.2													
HSC7E127	7q31.2													
HSC7E146	7q31.2													
HSC7E39	7q31.2													
HSC7E582	7q31.3													
cos 375	7q31.3-32.1													
HSC7E256	7q31.3													
HSC7E619	7q31.3													
HSC7E448	7q31.3													
HSC7E451	7q31.3													
HSC7E107	7q31.3													
HSC7E1289	**7q31.3-7q32**													
PAC54P14	**7q32**													
HSC7E125	**7q32**													
HSC7E195	**7q32**													
HSC7E648	7q33													
HSC7E1175	7q33													
HSC7E87	7q34													
CEPH 790h2	7q34													
HSC7E273	7q34													
HSC7E164	7q34													
CEPH 940a12	7q34													
cos 737	7q34-35													
CEPH 761h5	7q35													
CEPH 846 e 8	7q35													
CEPH 803g1	7q35													
CEPH 647f12	7q35													
cos 595	7q35-36													

Retained on der(7)
Deleted from der(7)
Diminished signal
Not Done

Bold border indicated the limits of the common deleted region, *case R017 had two abnormal homologs of chromosome 7, R017a and b, probes, the names of which are aligned to the right map within the probe directly above aligned to the left.

TABLE 5. LOH Results



ization with the 1800-kb YAC HSC7E1289 in the case 207/99 resulted in a diminished signal on the der(7), the size of which could be estimated as half of the signal seen on the normal homolog of chromosome 7.

Only four cases presented with interstitial deletions, allowing for the identification of the distal breakpoint, which was more uniform than the proximal one. In the other nine cases, the loss of chromosome 7 sequences was caused by either unbal anced translocation or by terminal deletion, with loss of all sequences telomeric to the identified breakpoints. One case (R017) with +del(7), del(7) had two abnormal homologs of chromosome 7 in addition to one normal, with breakpoints at 7q22 and 7q31 (Fig. 1). Therefore, although this case had a partial trisomy of chromosome 7, sequences telomeric to 7q32 were lost from the tumor cells. In summary, the FISH-CDR spanned from 7q32 to 7q33 and was about 12 Mb in size.

LOH

Allelic losses were readily recognizable by Genescan analysis, given that one of the alleles was nearly completely absent and there were no allelic imbalances manifesting themselves as a lower peak. Detailed results are presented in Table 5 and in Figure 3. No abnormalities were identified in the control marker (*D7S503*) mapping to the short arm of chromosome 7. All patients were informative for a minimum of 11/50 (22%) markers. Forty-two out of 50 (84%) markers were found to exhibit

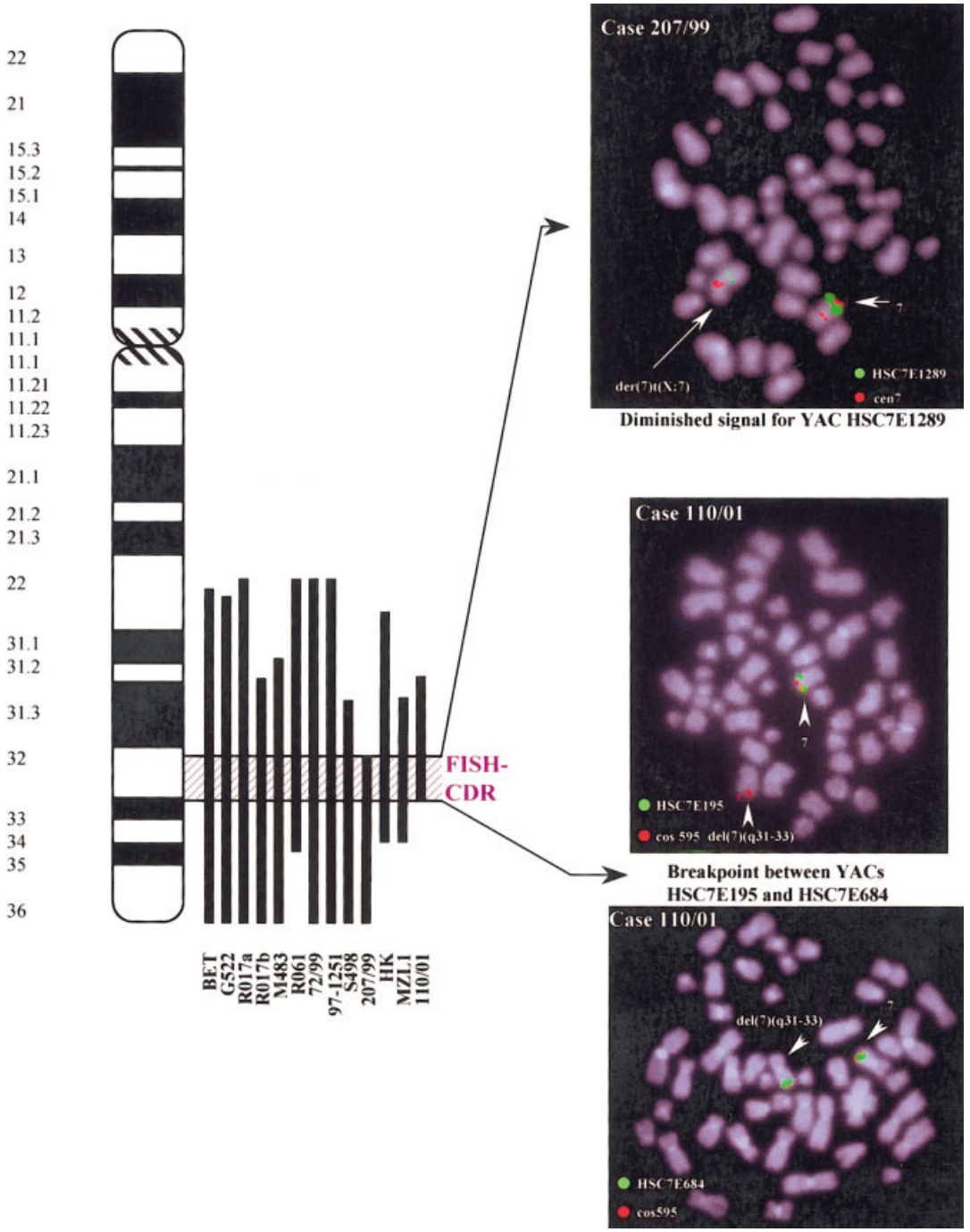


Figure 1. Results of the CDR mapping by metaphase FISH. The size of deletion identified in each patient was plotted against the ideogram of chromosome 7. The panel on the right-hand side shows FISH images from patients who allowed the identification of FISH-CDR.

LOH in at least one case. Six cases (20%) retained heterozygosity in all markers for which they were informative. Seven markers were uninformative in all 29 patients. The loss of the long arm of chromosome 7 from 7q22 was identified in one case in which conventional cytogenetics failed (patient 3). Two cases with small deletions within 7q31 were identified (patients 16 and 27). Twenty out of 21 (95%) cases with LOH showed the presence of noncontiguous deletions within the region studied.

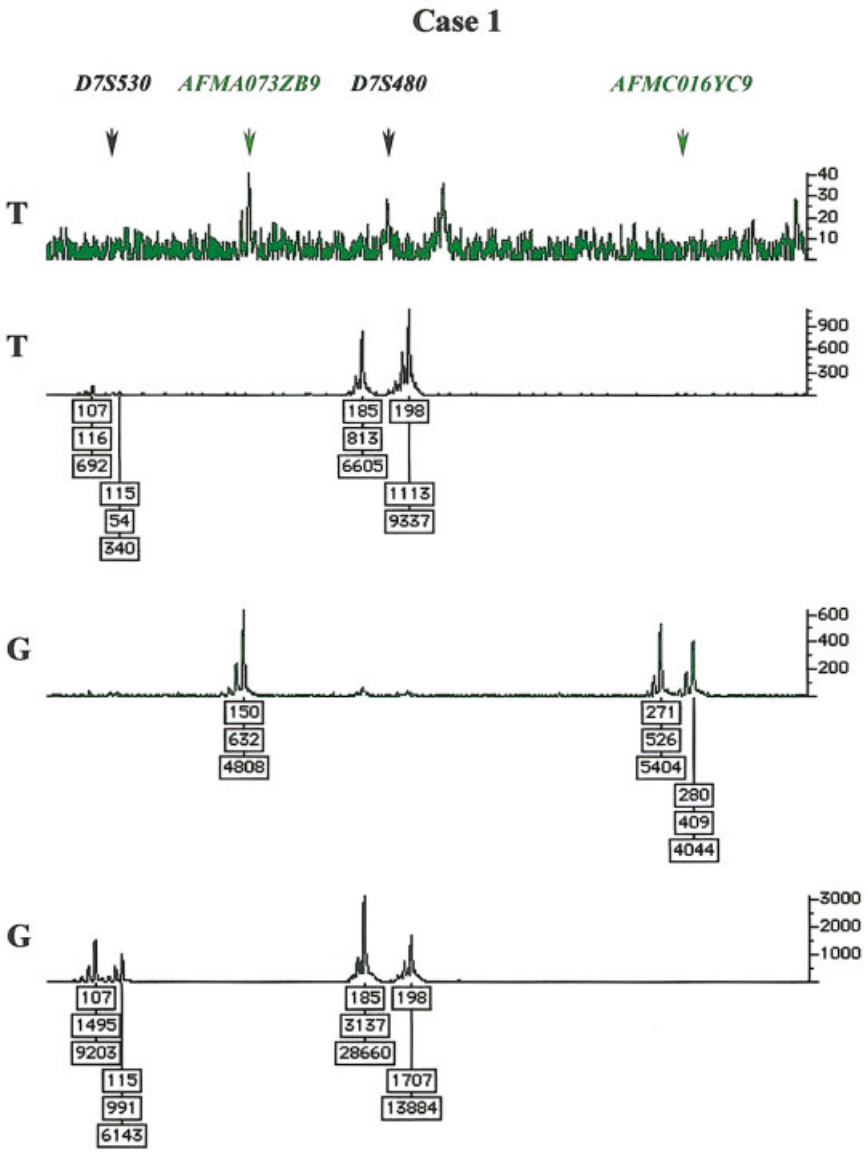


Figure 2. Example of a genotyping profile from a patient with biallelic deletion. Multiplex PCR reaction for markers *AFMA073ZB9* and *D7S480* and markers *AFMC016YC9* and *D7S530* in a patient with biallelic deletion. All markers in germline DNA (G) sample and *D7S480* and *D7S530* in the tumor DNA (T) sample were amplified, and the allele sizes obtained in multiplex PCR were the same as when the markers were amplified separately.

In three cases, DNA from two abnormal tissues, peripheral blood and spleen, was available and the results of LOH analysis on these tissues were concordant.

By use of a set of 50 microsatellite repeat markers for LOH study on a series of 32 samples from 29 cases, four regions with high frequency (>25%) of mono- or biallelic loss were identified (Table 5, Figs. 2 and 3). These regions encompassed the area of *D7S633– AFMA073ZB9*, the region between the *AFMC016YC9* and *D7S2544* marker *D7S2452*, and marker *AFMA129WE9*. The identified regions were, respectively, 8.21, 3.79, and 3.7 Mb apart; the size of the most centromeric hotspot was 0.95 Mb and of the second from the centromere was 0.85 Mb. The remaining telomeric hotspots were identified by a single microsatellite each.

The most frequent loss of both alleles occurred at the marker *AFMA073ZB9*; in 11/25 (44%) informative patients, neither allele was detectable by PCR amplification in tumor samples, although they were readily evident in the germline DNA samples. A further 8% of cases showed the presence of LOH in this marker, making up the abnormality rate at *AFMA073ZB9* to 52%. The second most frequently lost marker in both alleles was the *AFMC016YC9*, not detectable in tumor samples from 9/25 (36%) informative cases and with additional 8% of patients displaying LOH at this site. The marker with the highest incidence of LOH

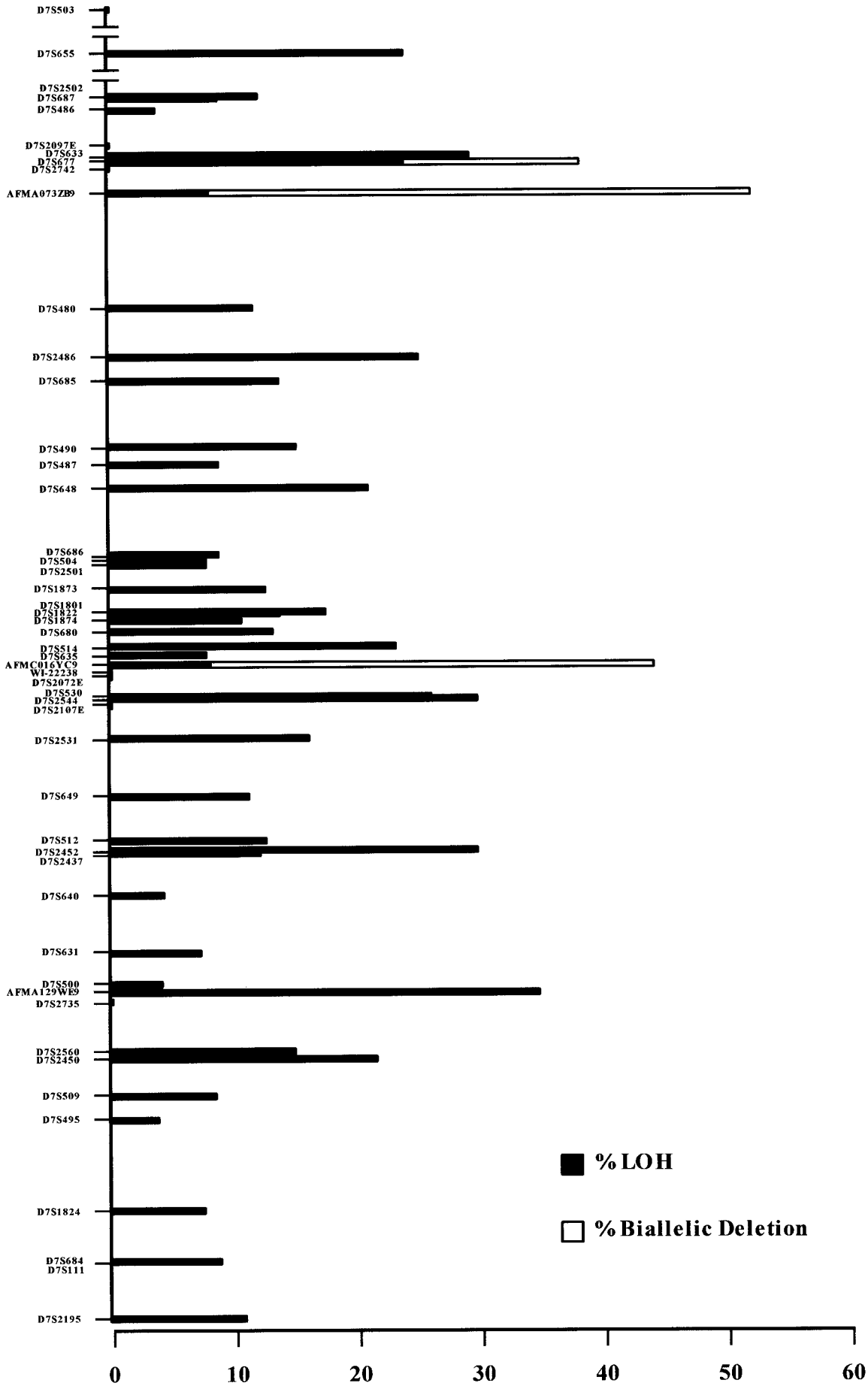

D7S503
D7S655
D7S2502
D7S687
D7S486
D7S2097E
D7S633
D7S677
D7S2742
AFMA073ZB9
D7S480
D7S2486
D7S685
D7S490
D7S487
D7S648
D7S686
D7S504
D7S2501
D7S1873
D7S1801
D7S1822
D7S1874
D7S680
D7S514
D7S635
AFMC016YC9
WI-22238
D7S2072E
D7S530
D7S2544
D7S2107E
D7S2531
D7S649
D7S512
D7S2452
D7S2437
D7S640
D7S631
D7S500
AFMA129WE9
D7S2735
D7S2560
D7S2450
D7S509
D7S495
D7S1824
D7S684
D7S111
D7S2195
% LOH
% Biallelic Deletion
0
10
20
30
40
50
60

Figure 3.

was *AFMA129WE9*, with LOH identified in 9/26 (34.6%) informative patients.

Experiments multiplexing each of the biallelically deleted markers with another marker with the same annealing temperature were conducted. There was amplification of this other marker in samples in which biallelic deletion was detected. PCR reactions that used corresponding germline DNA resulted in amplification of both markers (Fig. 2).

DISCUSSION

Chromosomal deletion, or, more precisely, gene deletion, contributes to carcinogenesis principally through the consequential loss of tumor-suppressor genes. There are several consistently deleted regions in B-cell malignancies, including 1p, 3p, 3q, 6q, 7q, 10q, 11q, 13q, and 17p. Isolation and functional characterization of genes within the deleted regions may provide insights into the biology and clinical behavior of B-cell malignancies.

Abnormalities of chromosome 7 constitute around 26% of the abnormal karyotypes in SLVL (Oscier et al., 1993, 1996). However, chromosome 7 aberrations are not pathognomonic for this disease and have been described in a variety of B-cell disorders (Sole et al., 1993; Offit et al., 1995; Oscier et al., 1996; Hernandez, 1997a; http://cgap.nci.nih.gov/Chromosomes/Mitelman) as well as in other hematological malignancies, particularly of mye loid origin (http://cgap.nci.nih.gov/Chromosomes/Mitelman). This recurrent loss of material from chromosome 7 in disparate tumors suggests that there may be as yet uncharacterized tumor-suppressor gene or genes harbored in this area. This notion is further supported by a high incidence of chromosome 7 abnormalities in solid tumors, although no gene(s) has as yet been identified for this tumor group (http://cgap.nci.nih.gov/Chromosomes/Mitelman).

In the present study, FISH was used to map the CDR on the long arm of chromosome 7, in a series of patients with SLVL displaying aberrations of this chromosome. The analysis of LOH, a recognized approach for identification of areas within the genome where putative tumor-suppressor genes may be located, can be genome-wide or, as in the case of this project, selective to a particular chromosomal region. In this study, the use of microsatellite markers, subsequent to identification of the FISH-CDR on the long arm of chromosome 7, allowed for further narrowing of the disease loci within this chromosomal region. LOH analysis in a series of well-characterized patients with SLVL led to identification of four distinct loci with high frequency of abnormalities and retention of sequences between them. These results may be consistent with the heterogeneous nature of SLVL with variable phenotype, cytogenetic abnormalities, and clinical behavior.

Similarly, two different regions of LOH on the long arm of chromosome 7 have been reported in leiomyomas (Ishwad et al., 1997) and ovarian cancer (Edelson et al., 1997) and three distinct areas in myeloid neoplasms (Liang et al., 1998). The pattern of deletions in the current study in individual patients was also nonlinear, with one or more heterozygous markers flanked by deleted microsatellites. This pattern has been observed by others in a variety of tumors with abnormalities of 7q and other chromosomes (Edelson et al., 1997; Ishwad et al., 1997; Zeng et al., 1997, 1999; Liang et al., 1998). Noncontiguous deletions in individual patients may reflect the presence of complex submicroscopic chromosomal abnormalities, chromosomal fragility, or the existence of multiple critical loci within the analyzed region. The heterogeneity of the LOH profile throughout the region studied here is striking, indicating general genomic instability/fragility and the possibility that the identified hotspots merely constitute extremes of this tendency; however, this is not the first report showing such a pattern of LOH (Liang et al., 1998). There were no abnormalities in the control 7p marker and no signs of microsatellite instability (e.g., different microsatellite size in the germline and tumor samples), and abnormalities within the hotspots were identified in a high proportion of patients, despite the small number of patients studied.

Losses of both alleles were identified for three markers. Regardless of the mechanism, biallelic loss strongly indicates that a given region is an important disease site and provides strong evidence for the existence of genes relevant for the pathogenesis of SLVL in the hotspot regions. Experiments with multiplex PCR showed that the lack of PCR amplification was specific to the three markers. Homozygous deletion was identified by Mateo and colleagues (1999) in 1/20 patients with splenic marginal zone lymphoma (SMZL) from their series. Biallelic deletions are a rare genetic event, leading to inactivation of genes.

Figure 3. Results of LOH experiments. The frequency of LOH and biallelic deletions per marker was plotted by use of the FigP program; the distances between markers are proportional to the physical distance between them, contained in Table 3.

The four loci identified in this study are different from the region identified by previous reports (Hernandez et al., 1997b; Mateo et al., 1999). Hernandez et al. (1997b) examined a group of unselected patients with B-NHL and used FISH probing with large CEPH YACs. Only six patients were examined, and not all probes were tested on all patients. Moreover, the probes used were noncontiguous, and deletion between the YACs would have remained undisclosed. Finally, resolution of FISH with such large probes is limited and small deletions of fragments of YACs may be missed. The authors identified a region of 5 cM between *D7S685* and *D7S514* (Hernandez et al., 1997b); hotspot regions identified here are located centromeric and telomeric to the one postulated by Hernandez and co-authors. Mateo et al. (1999) analyzed patients with SMZL with 13 markers from within chromosome arm 7q and used a radioactive LOH technique that might be difficult to interpret. The highest incidence of LOH was identified in the microsatellite *D7S487*, lost in 5/11 informative patients. This marker lies in the middle of the region identified by Hernandez et al. and therefore between the two main loci identified here.

Mateo and co-workers (1999) identified abnormalities in 8/20 (40%) SMZL patients studied, and 80% of cases from the current study had an abnormality within at least one marker. Only 3/8 patients with allelic losses from Mateo's series had LOH, one had both allelic imbalances and LOH, and a further four patients had allelic imbalances alone. No allelic imbalances were detected in the current series. The higher number of patients with abnormalities in the present study as well as the presence of LOH, and not allelic imbalances, may be explained by the fact that the specimens were enriched for tumor cells and therefore the content of normal cells in the patient's samples was reduced. Also, it is conceivable that with the increased sensitivity of fluorescent-LOH, more abnormalities were detected.

The most centromeric region of high frequency of losses identified by LOH is not included in the FISH-CDR. As shown in Table 4 and Figure 1, 7/12 cases showed a breakpoint within chromosomal band 7q22, and the long arm of chromosome 7 in a further case was breaking at the boundary between 7q22 and 7q31; in these eight cases, the sequences from within the centromeric biallelic deletion/LOH hotspot were deleted. Thus, only three cases from the series studied by FISH did not lose the chromosomal segment in question. The discrepancy between FISH and LOH results may have arisen from:

1. A relatively small series of patients was included for the identification of FISH-CDR.
2. Only 4/13 abnormal chromosomes 7 studied presented with interstitial deletion; a possibility thus exists that, if a larger cohort and more patients with interstitial losses were identified for FISH mapping of CDR, two chromosomal areas would have been identified: one at 7q31 and one at 7q32–33.
3. The deletion at 7q31 is small, discrete, and noncontiguous and the resolution of FISH is not high enough to identify it.
4. Because the YACs used were not forming a contig, a deletion between clones used would be missed with this probe set.
5. YAC HSC7E127 contains the marker *D7S677*, in which a biallelic deletion was found in three cases. The cases studied by FISH may not have had a deletion in this marker (the deletion was not universally present in all cases studied by LOH). Because this YAC is 560 kb in size and the *D7S677* is 280 bp, a small interstitial deletion would remain silent when tested with this YAC probe.
6. The FISH part of this project identified the CDR; all patients with chromosome 7 abnormalities studied lost the sequences from within this region; the LOH hotspots were not common for all patients, but were the most frequently affected sites among markers examined.
7. Perhaps a deletion of one area of the four identified by LOH is sufficient to confer the disease phenotype.

In conclusion, we have identified CDR by FISH on the long arm of chromosome 7 to 7q32–33 on a series of 13 patients with cytogenetically abnormal chromosomes 7. Further study by fluorescent LOH recognized four areas of high incidence of biallelic deletions and LOH. Additional studies have to be conducted to identify candidate genes important for the initiation of this disease.

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