

PLAG1 Alterations in Lipoblastoma

Involvement in Varied Mesenchymal Cell Types and Evidence for Alternative Oncogenic Mechanisms

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Lipoblastomas are rare soft tissue tumors that occur primarily in young children. They typically contain variably differentiated adipocytes, primitive mesenchymal cells, myxoid matrix, and fibrous trabeculae. Abnormalities in chromosome 8, leading to rearrangements of the *PLAG1* gene, were demonstrated recently in four lipoblastomas. In the present report, we determine the frequency of *PLAG1* alterations in 16 lipoblastomas from children aged 13 years or younger, and we also evaluate the stages of lipoblastoma differentiation at which *PLAG1* genomic alterations are found. Eleven lipoblastomas (69%), including those with either classic or lipoma-like histology, had rearrangements of the 8q12 *PLAG1* region. Another three lipoblastomas had polysomy for chromosome 8 in the absence of *PLAG1* rearrangement. Only two cases (13%) lacked a chromosome 8 abnormality. Notably, the lipoblastomas with chromosome 8 polysomy had up to five copies of chromosome 8 as an isolated cytogenetic finding in an otherwise diploid cell. We also demonstrate that *PLAG1* alterations are found in a spectrum of mesenchymal cell types in lipoblastomas, including lipoblasts, mature adipocytes, primitive mesenchymal cells, and fibroblast-like cells. This finding is consistent with neoplastic origin in a primitive mesenchymal precursor and with variable differentiation to a mature adipocyte end-point. Hence, our studies provide biological validation for the clinical observation that lipoblastomas can evolve into mature, lipoma-like, lesions. They

also suggest that *PLAG1* dosage alterations caused by polysomy 8 might represent an alternative oncogenic mechanism in lipoblastoma. (Am J Pathol 2001, 159:955-962)

Lipoblastomas are fatty tumors primarily affecting children younger than 3 years of age.^{1,2} Most lipoblastomas present in the superficial tissues of the arms and legs, but they may also arise in the head and neck regions, trunk, mediastinum, and retroperitoneum. Although lipoblastomas do not metastasize, they can recur locally after incomplete excision. Lipoblastomas feature a wide spectrum of adipocytic differentiation, in which mono- and multivacuolated lipoblasts are admixed with mature adipocytes. The adipocytic cells are often set in a variably myxoid matrix containing spindle or stellate mesenchymal cells, and the tumor is usually organized into lobules, separated by fibrous septae. The admixture of lipoblasts and mature adipocytes has given rise to the broadly held notion that lipoblastomas can mature to simple lipomas.³ Recently, we reported chromosomal rearrangements involving band 8q12 in four lipoblastomas. Each of these alterations targeted the *PLAG1* oncogene, which encodes a zinc finger transcription factor, expressed primarily in fetal tissues and only at very low levels postnatally.^{4,5} Notably, the 8q12 rearrangements in lipoblastoma were associated with promoter swapping, in which the *PLAG1* promoter element was replaced by those from the hyaluronic acid synthase 2 (*HAS2*) or collagen (*COL1A2*) genes. These promoter swapping events accomplish *PLAG1* transcriptional up-regulation by substitution of an active promoter and are mechanistically similar to the *PLAG1* rearrangements in pleomorphic adenomas of salivary gland.⁴ In the present study, we sought to further characterize the role of *PLAG1* alteration in lipoblastoma by determining the frequency of genomic *PLAG1* rearrangements and by

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Table 1. Clinical, Histopathological, Cytogenetic, and *in Situ* Hybridization Data for 16 Lipoblastomas

Case	Histology*	Age/sex	Location	Cytogenetics	<i>In situ</i> hybridization
1	Classic	2.5/M	Perineal	8q12 rearrangement	<i>PLAG1</i> split
2	Classic	1.5/F	Back	8q12 rearrangement	<i>PLAG1</i> split
3	Classic	7/M	Intramuscular	8q12 rearrangement	<i>PLAG1</i> split
4	Classic	6.5/M	Mediastinal	Ring chromosome 8	<i>PLAG1</i> split
5	Classic	1/M	Subscapular	8q12 rearrangement	<i>PLAG1</i> split
6	Classic	3.5/F	Neck	8q12 rearrangement	<i>PLAG1</i> split
7	Classic	13/M	Hip	8q12 rearrangement	<i>PLAG1</i> split
8	Classic	1/M	Axilla	8q12 rearrangement	<i>PLAG1</i> split
9	Classic	1.5/F	Hip	Normal	Disomy 8, tetrasomy 8
10	Classic	5/M	Back	Normal	Disomy 8
11	Classic	2/F	Leg	Normal	Disomy 8
12	Myxoid	1.5/M	Neck	8q12 rearrangement	<i>PLAG1</i> split
13	Myxoid	7/M	Neck	Pentasomy 8	Disomy 8, trisomy 8, tetrasomy 8, pentasomy 8, hexasomy 8
14	Lipoma-like	5.5/F	Arm	8q12 rearrangement	<i>PLAG1</i> split
15	Lipoma-like	8 mo/F	Neck	Normal	<i>PLAG1</i> split
16	Hibernoma-like	1/M	Back	Not Done	Disomy 8, trisomy 8, tetrasomy 8

*Histological subclassification is defined in the text.

evaluating *PLAG1* alterations in cells at different levels of differentiation.

Materials and Methods

Patients

Cytogenetic and molecular genetic data on cases 1 to 5 and 8 have been previously published.⁵⁻⁷ Clinical and histopathological data for all cases are found in Table 1.

Chromosome Analysis

Metaphase harvest and slide preparation from tumor cell cultures were as described previously,⁸ with minor modifications. G banding was obtained by trypsin-Giemsa staining. For fluorescence *in situ* hybridization, chromosome preparations were pretreated in 2× standard saline citrate at 37°C for 1 hour, followed by 5 minutes of digestion with pepsin using 1:25 diluted Digest-All III (Zymed Laboratories, South San Francisco, CA). Biotinylated and digoxigenin-labeled α satellite probes for the centromeric regions of chromosomes 4, 8, 11, and 12 were from Oncor (Gaithersburg, MD). The yeast artificial chromosome probes 935e9 and 946b7, flanking *PLAG1*, were obtained from Center d'Etude Polymorphisme Humain, Paris, France. Yeast artificial chromosome DNA was isolated as previously described⁸ and labeled by either digoxigenin or biotin by random octamer priming, using the BioPrime DNA labeling system (Life Technologies Inc., Rockville, MD). After labeling, DNA was purified by S-200HR spin column chromatography (Pharmacia, Uppsala, Sweden), co-precipitated with 5× Cot-1, and resuspended in hybridization buffer (50% formamide, 10% dextran sulfate, 2× standard saline citrate). Probes and chromosome preparations were co-denatured at 75°C for 2 minutes and hybridized overnight. Biotin- and digoxigenin-labeled probes were detected by streptavidin-Alexa-498 (Molecular Probes, Eugene, OR) and fluorescein anti-

digoxigenin (Roche Diagnostics, Indianapolis, IN), respectively. Chromosomes were counterstained using diaminodiphenylindole (Vector Laboratories, Burlingame, CA).

Genetic Analyses of Tissue Sections

Chromogenic *in situ* hybridization (CISH) was performed on 4- μ m formalin-fixed, paraffin-embedded, tissue sections on positively charged slides (SuperFrost Plus; Menzel-Gläser, Germany). The slides were baked overnight at 65°C and then deparaffinized in xylene for 3 × 15 minutes, followed by washing in 100% ethanol for 2 × 2 minutes. Air-dried slides were treated in 100 mmol/L Tris/50 mmol/L ethylenediaminetetraacetic acid (pH 7.0) in a microwave oven at 99°C for 15 minutes, washed briefly in phosphate-buffered saline (PBS), and digested with Digest-All III (Zymed Laboratories) for 1 to 7 minutes. The duration of the pepsin digestion was optimized for each case to obtain sufficient signal intensity without loss of tissue architecture. After washing in PBS, the slides were immersed in 10% formalin/PBS for 1 minute and dehydrated for 4 × 2 minutes in a 70 to 85% to 95 to 100% ethanol series. Probes for CISH were labeled with digoxigenin and fluorescein (New England Nuclear, Boston, MA), by random octamer priming, as described above. Tissue sections and probes were co-denatured at 94°C for 3 minutes and hybridized overnight at 37°C. Probe detection was with alkaline phosphatase anti-digoxigenin (Roche Molecular Biochemicals, Indianapolis, IN) and Fast Red substrate, and with horseradish peroxidase anti-fluorescein and H₂O₂-diaminobenzidine (Zymed Laboratories). Tissue sections were counterstained with hematoxylin and mounted with Glycerogel (DAKO, Carpinteria, CA). At least 50 cells (mean, 150) were analyzed from each tumor. Tumors with >10 cells showing one red-brown intact signal, one separate red, and one separate brown signal at yeast artificial chromosome probe hybridization were scored as positive for

PLAG1 rearrangements. Tumors with >10 cells showing >2 signals for centromere 8 and ≤ 2 signals with a centromeric probe for another chromosome were scored as polysomic for chromosome 8.

Results

Histopathology

Review of the histology slides from the 16 lipoblastomas showed that all tumors were characterized by lobules of mature and immature fat cells including lipoblasts of the monovacuolated and multivacuolated variety. The fibrous septae varied in thickness and consisted of delicate and/or wiry collagen with interspersed spindle, fibroblast-like cells, some of which had plump nuclei and abundant cytoplasm and sometimes very small cytoplasmic vacuoles. There was substantial difference in the histological appearance of some tumors, prompting the classification described below. The classic lipoblastomas ($n = 11$; Table 1) usually had a minimal myxoid component—limited to rare microscopic foci in some tumors—consisting of intercellular mucin, spindle cells, and stellate primitive mesenchymal cells, which blended with the adipocytic component (Figure 1A). The myxoid lipoblastomas ($n = 2$) contained abundant interstitial mucin that comprised >50% of the specimen (Figure 1B). The lipoma-like lipoblastomas ($n = 2$) lacked a myxoid component and were composed predominantly of mature adipocytes with scattered monovacuolated and multivacuolated lipoblasts (Figure 1C). The last tumor also lacked a myxoid component and was composed predominantly of multivacuolated lipoblasts, some of which had central nuclei and granular eosinophilic cytoplasm. This tumor was designated a “lipoblastoma with hibernoma-like features” (Figure 1D). All tumors were categorized using the above-mentioned descriptors (Table 1).

G Band and Fluorescence in Situ Hybridization/CISH Analyses

G-banding analyses revealed rearrangement of the 8q12 chromosome region in 9 of 16 lipoblastomas, and a ring chromosome 8 with unassignable cytogenetic breakpoints in the chromosome 8 long arm in another tumor (Table 2). PLAG1 abnormalities were evaluated in all 16 tumors by metaphase cell fluorescence *in situ* hybridization (12 cases) and/or paraffin-section interphase cell CISH (10 cases). CISH was used for the paraffin sections because lipid-related autofluorescence interfered with fluorescence *in situ* hybridization detection methods, particularly when attempting to evaluate the adipocytic populations in the sections (data not shown). After optimization of the enzymatic digestion procedure, the paraffin sections from each of the 10 lipoblastomas could be analyzed with a simultaneous appreciation of tissue architecture, cellular morphology, and chromosomal abnormalities. The PLAG1 *in situ* hybridization analyses confirmed rearrangement of the PLAG1 region (Figure 2, a

and b) in all lipoblastomas with cytogenetic 8q rearrangement and in one lipoblastoma that had been diploid cytogenetically. In addition, the *in situ* hybridization analyses revealed three to six copies of chromosome 8 in three lipoblastomas, including one (case 13) that had pentasomy 8 by G banding (Figure 3). Neither chromosome banding nor molecular cytogenetic analyses showed evidence of a general increase in DNA ploidy in the polysomy 8 lipoblastomas. Not only was the karyotype indicative of an isolated chromosome 8 polysomy in case 13, but there were no polysomies for four comparison chromosomes (1, 4, 11, and 12, by α -satellite probes) in any of the polysomy 8 tumors (Figure 2c). None of the cells in the polysomy 8 tumors had molecular cytogenetic evidence of PLAG1 rearrangement.

Notably, PLAG1 rearrangement or copy number gain was found in lipoblastomas irrespective of histological subtype (Table 1). In particular, PLAG1 alterations were demonstrated in each of two cases, which were composed predominantly of mature adipocytic cells (lipoma-like lipoblastoma). Six lipoblastomas, including three with PLAG1 rearrangement (cases 1, 7, and 12) and three with PLAG1 copy number gain (cases 9, 13, and 16), were evaluated to determine which cell types contained the genomic alterations. All six tumors contained monovacuolated and multivacuolated lipoblasts as well as more mature adipocyte-like cells. PLAG1 rearrangement was demonstrated in each of the mesenchymal cell components—lipoblastic, mature adipocytic, stellate primitive mesenchymal cell, and fibroblast-like cell—in these tumors. However, PLAG1 was not rearranged in the vascular elements, including endothelial cells and leukocytes, within the tumor parenchyma. Similarly, the three lipoblastomas with polysomy 8 contained additional PLAG1 signals in both the lipogenic cells and fibroblast-like cells, including those in fibrous septae (Figure 2, d and e), but not in the vessels (Figure 2e). The size of the polysomic cell populations varied in these tumors. Case 13 had three to six copies of chromosome 8 in all cells of the tumor, except for the vessels, whereas cases 9 and 16 had localized cell populations with three or four copies of chromosome 8.

Discussion

Most lipogenic tumors exhibit characteristic chromosomal rearrangements, which are associated with rearrangement and/or altered expression of specific oncoproteins. Myxoid liposarcomas typically have chromosomal abnormalities involving band 12q13, resulting in rearrangement of the CHOP gene with either FUS in 16p11 or EWS in 22q12.^{9,10} The FUS-CHOP and EWS-CHOP fusion oncoproteins are transcriptional regulators, affecting the expression of various genes associated with adipocytic differentiation.^{11,12} Well-differentiated liposarcomas, on the other hand, are characterized by supernumerary ring and giant marker chromosomes, containing amplified sequences from the long arm of chromosome 12.^{13,14} These amplicons generally include the MDM2 oncogene, the product of which inhibits normal TP53 function and

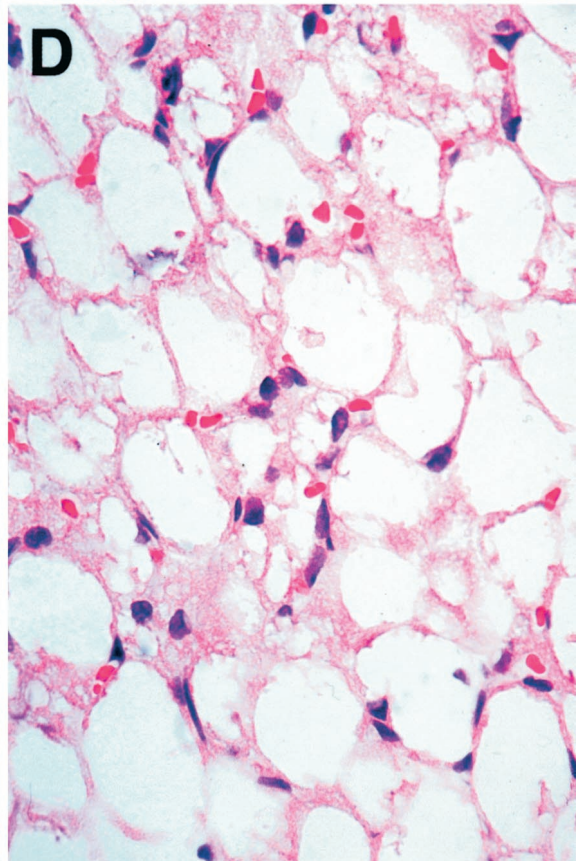
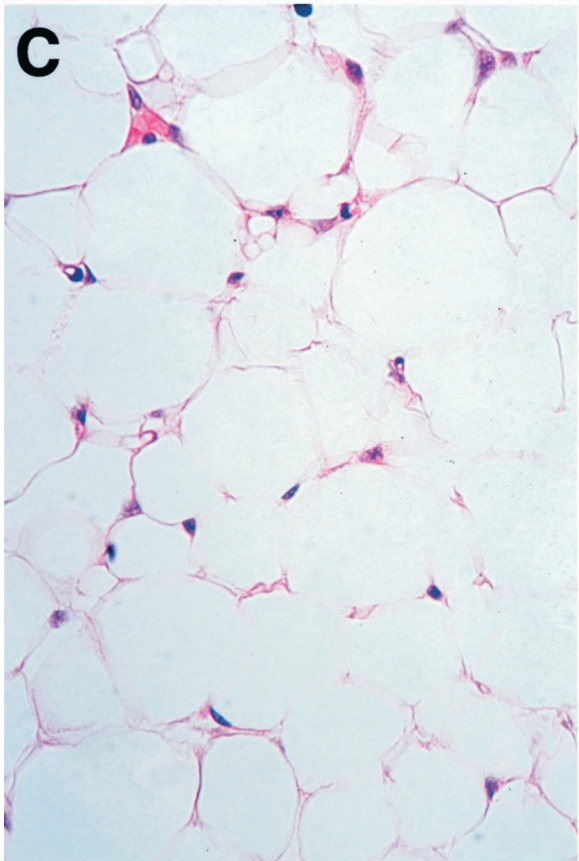
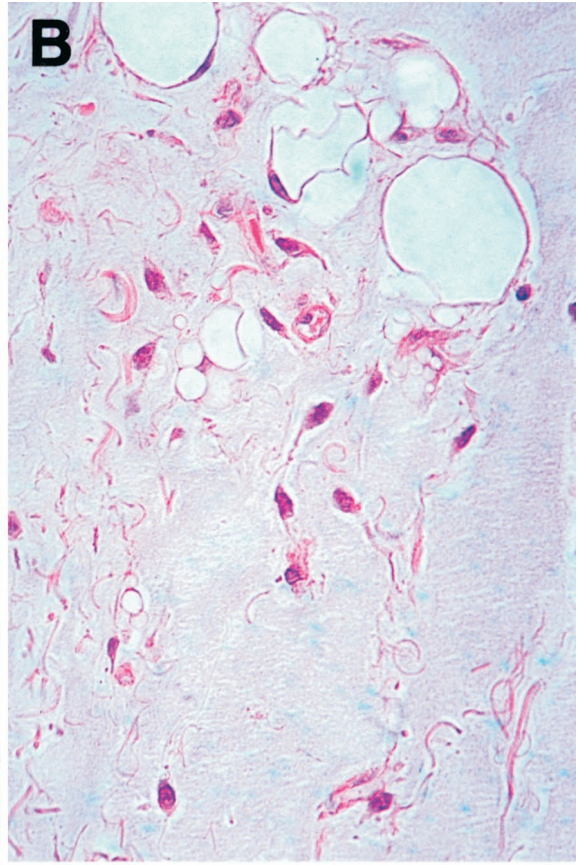
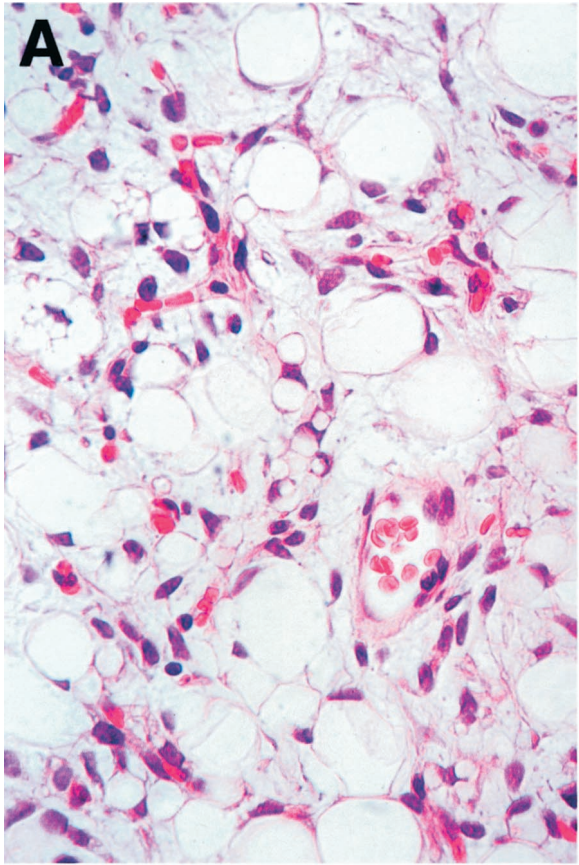


Table 2. G-Banded Karyotypes for 16 Lipoblastomas

Case	Karyotype
1	46,XY,ins(6;8)(p25;q12q24.1)*†
2	46,XX,der(1)(7pter->7p22::8q24.1->8q22::1p13->1qter),der(7)(1pter->1p13::8q22>8q13::7p22->7qter),der(8)(pter->q13::q24.1->qter)**†
3	46,XY,t(8;12)(q12;q24)
4	46,XY,r(8)*
5	46,XY,del(6)(q11),der(7)t(6;7)(q11;p22),t(7;8)(q22;q12),del(8)(q12)*
6	47,XX,del(3)(q12),-8,+der(?)t(8;?)(q12;?),+mar1
7	46,XY,t(1;8)(p22;q12)
8	46,XX,t(8;9)(q12;p22)‡
9	46,XX
10	46,XY
11	46,XX
12	46,XY,t(3;8)(q12;q12)
13	49,XY,+8,+8,+8
14	46,XX,del(2)(q23q31),der(5)t(5;8)(p11;q12),der(8)(8pter->8q12::?:5p11->5pter)
15	46,XX
16	Not done

Previously published by *Hibbard et al.,⁵ †Fletcher et al.,⁶ and ‡Dal Cin et al.⁷

thereby promotes cell cycle progression.^{15,16} Nonrandom chromosomal alterations are also found in benign adipocytic tumors. Approximately 60% of typical, mature, lipomas have rearrangements of the *HMGIC* locus at chromosome band 12q15.¹⁷⁻¹⁹ *HMGIC* encodes a DNA-binding protein, expressed primarily in embryogenic tissues, the functions of which include regulation of mesenchymal cell growth and differentiation.²⁰ A similar situation exists in lipoblastoma, where 8q12 chromosomal rearrangements result in up-regulated expression of the *PLAG1* transcription factor.^{5,21}

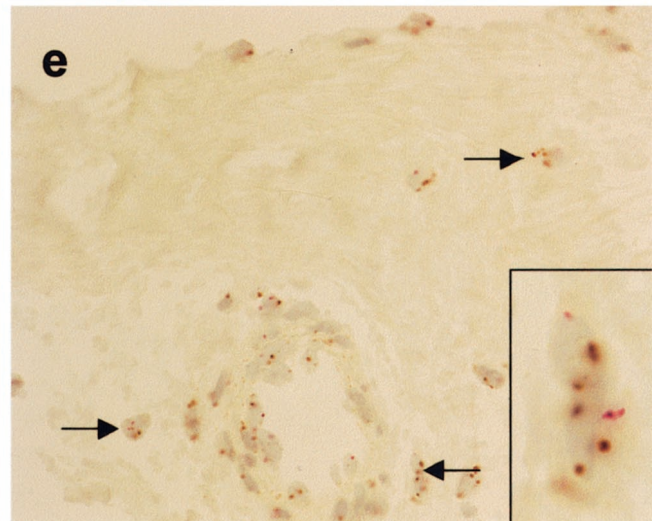
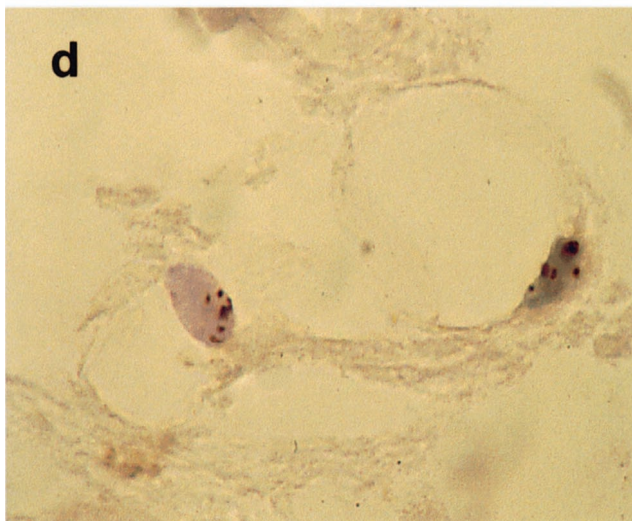
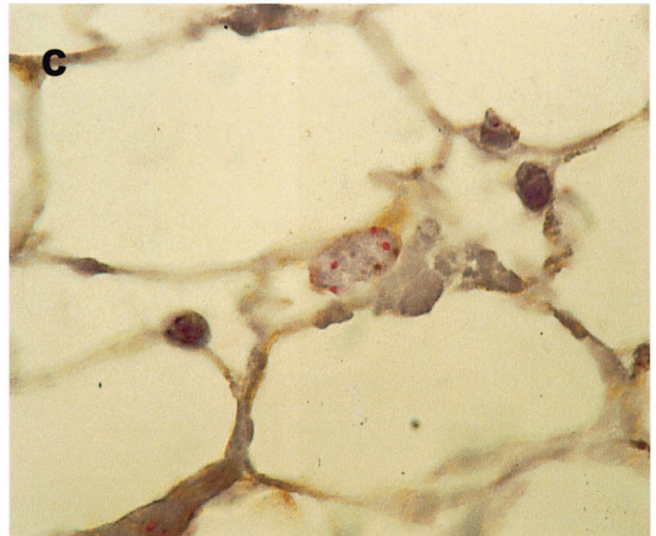
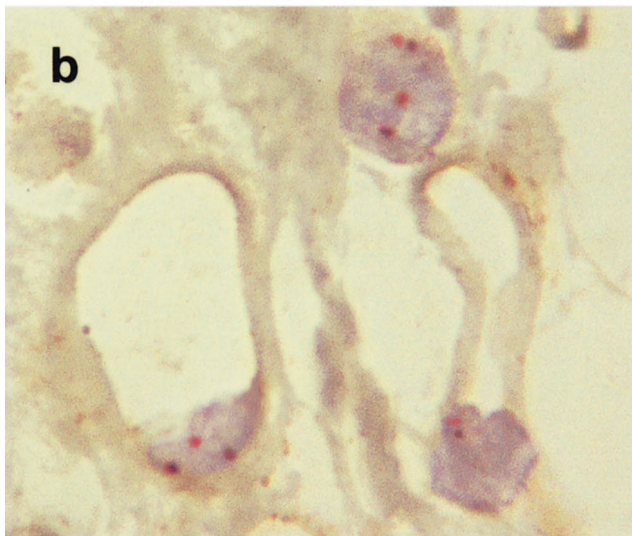
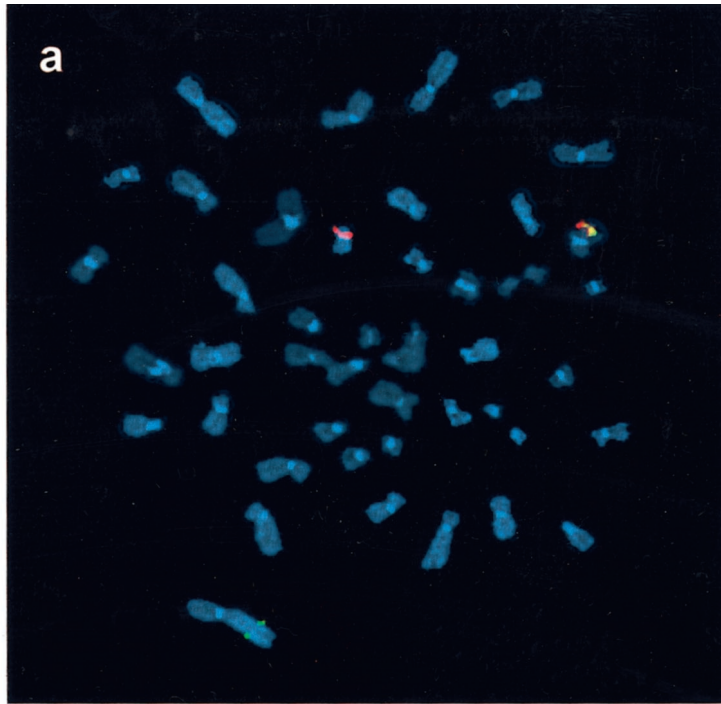
In the present study, we found *PLAG1* rearrangements in 11 of 16 lipoblastomas, and we also showed that the *PLAG1* nonrearranged tumors may have novel oncogenic mechanisms involving *PLAG1* copy number gains. These data suggest an either/or relationship between *PLAG1* rearrangement and *PLAG1* amplification in lipoblastoma tumorigenesis. An analogous situation has been observed in uterine leiomyomas, where ~25% (80 of 325) of tumors contain rearrangements of the *HMGIC* region in 12q15, whereas another 10% (27 of 325) are trisomic for chromosome 12 (Mitelman F, Johansson B, Mertens F: Mitelman Database of Chromosome Aberrations in Cancer. <http://cgap.nci.nih.gov/Chromosomes/Mitelman>. 2001). Although *PLAG1* expression has not been compared in lipoblastomas containing *PLAG1* rearrangements versus amplifications, we hypothesize that *PLAG1* transcriptional up-regulation will be a consistent feature in both groups. Moreover, it is possible that the low-level amplified *PLAG1* loci are not wild type. Rather, these loci might contain point mutations within binding sites of *PLAG1* transcriptional repressors, which would thereby contribute to *PLAG1* transcriptional up-regulation. There is precedent for promoter-region point mutations in association with nonfusion *MYC* and *BCL6* oncogenes in Burkitt lymphomas²² and in follicular center cell lymphomas,²³ re-

spectively. However, it is yet known whether analogous genomic mechanisms contribute to transcriptional up-regulation in nonlymphoid tumors.

Most lipoblastomas contain primitive mesenchymal cells and adipocytic cells at various stages of maturation.¹ Typically, these cells are arranged in lobules showing a pattern of gradual differentiation, with the immature mesenchymal cells and multivacuolated lipoblasts at the periphery and the monovacuolated lipoblasts and adipocytes toward the center. This organization recapitulates the architecture of embryonal fat and it has been shown that some lipoblastomas eventually evolve into mature fat, presumably by terminal differentiation.^{3,23-26} If this is the case, one would anticipate a lipoma-like morphology in some lipoblastomas. Indeed, in the present study, two lipoma-like lipoblastomas were shown to contain *PLAG1* rearrangements. However, it is unlikely that any substantial number of adult lipomas result from lipoblastoma maturation. Adult lipomas are vastly more common than lipoblastoma, and they are characterized by a cytogenetic profile very different from that in lipoblastoma. Previous cytogenetic data have shown that only 1% (2 of 216) of lipomas from patients aged 15 years or older contain 8q11-13 rearrangements (Mitelman F, Johansson B, Mertens F: Mitelman Database of Chromosome Aberrations in Cancer. <http://cgap.nci.nih.gov/Chromosomes/Mitelman>. 2001). The frequency of trisomy 8 in these tumors is 2% (4 of 216). This paucity of lipoma *PLAG1*-region rearrangements is striking, particularly because—as discussed below—we demonstrated *PLAG1* rearrangements in the lipoma components of lipoblastomas. It follows that lipoblastomas and lipomas generally arise by distinct tumorigenic mechanisms, and possibly from different adipocytic progenitor cells.

CISH analysis of paraffin sections in six lipoblastomas revealed that the *PLAG1* alterations were present in lipo-

Figure 1. Photomicrographs of representative lipoblastoma histologies. **A:** Classic lipoblastoma has stellate and spindle mesenchymal cells, monovacuolated and multivacuolated lipoblasts, adipocytes, and intercellular mucin. **B:** Myxoid lipoblastoma is characterized by abundant intercellular mucin. **C:** Lipoma-like lipoblastoma has mostly mature adipocytes with only a rare lipoblast. **D:** Hibernoma-like lipoblastoma has polyvacuolated adipocytes with granular cytoplasm with some cells having central nuclei.



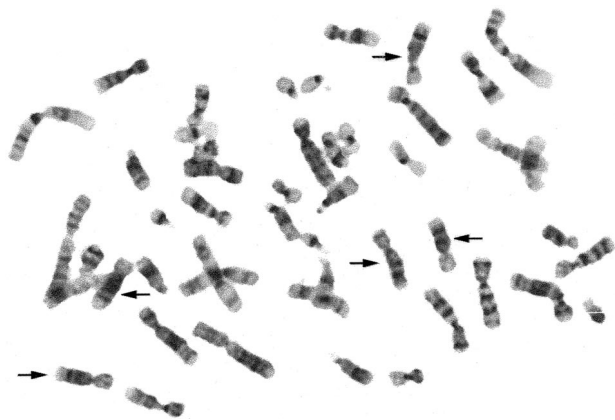


Figure 3. Metaphase cell from lipoblastoma case 13, stained by trypsin-Giemsa. **Arrows** indicate the five copies of chromosome 8.

blasts, mature adipocytes, stellate primitive mesenchymal cells, and fibroblast-like cells. The presence of *PLAG1* rearrangements in variably differentiated mesenchymal cells suggests an origin from a transformed mesenchymal progenitor that proliferates and differentiates in a manner similar to white fat. Because lipoblastomas occur in children primarily younger than 10 years of age, we expect that such progenitors are only present, or only susceptible to *PLAG1*-mediated neoplastic transformation, during the first few years of life. Such a window of vulnerability in cellular development has been suggested in connection with other neoplasms, in particular hematological malignancies. However, the difficulties in determining histological origin of tumor cell populations have left this hypothesis primarily unsubstantiated in solid neoplasms.²⁷ Lipoblastomas provide a compelling example of such a scenario, and in contrast to leukemias, in which expression of fusion transcription factor oncogenes can block cell maturation,²⁸ lipoblastomas follow a remarkably well-ordered sequence of differentiation and remain benign. Much might be learned by characterizing the biological impact of abnormally expressed developmental factors, such as *PLAG1*, in benign tumors. In particular, it would be important to determine whether the benign nature of lipoblastomas is dictated by cell context (the specific progenitor cell) or whether this is a general aspect of *PLAG1*-mediated transformation.

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Figure 2. a: Fluorescence *in situ* hybridization on metaphase chromosomes with centromeric (red) and telomeric (green) *PLAG1* probes demonstrates *PLAG1* rearrangement in lipoblastoma case 2. **b:** CISH with *PLAG1* probes in paraffin section from lipoblastoma case 2: splitting apart of red and brown probe signals is seen in adipocytes. **c:** CISH with chromosome 8 (red) and chromosome 10 (brown) probes in paraffin section from lipoblastoma case 9: polysomy 8 is evident in the adipocytic component. **d** and **e:** CISH with chromosome 8 (brown) and chromosome 11 (red) probes in paraffin section from lipoblastoma case 13: polysomy 8 is evident in both adipocytic (**d**) and fibrous (**e**) components but not in vascular element (**e**).

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