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Localization of a Novel Tumor Suppressor Locus on Human Chromosome 3q Important in Osteosarcoma Tumorigenesis[1]

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ABSTRACT

Mitotic recombination or nondysjunction are common mechanisms for tumor-specific loss of constitutional heterozygosity (LOH) and tumor suppressor allelic inactivation and can be useful in localizing new putative tumor suppressor genes. In osteosarcoma, the highest frequencies of LOH have been reported for chromosomes 3q, 13q, 17p, and 18q. The high incidence of LOH on chromosome 3q suggests the presence of a novel tumor suppressor gene. To localize this putative tumor suppressor gene, we have used polymorphic markers on chromosome 3q to define the minimal region in which mitotic recombination or deletion results in LOH, which should contain the tumor suppressor gene. This putative tumor suppressor has been localized to a region between 3q26.2-3q26.3 of less than 1 cM between the polymorphic loci *D3S1212* and *D3S1246*.

INTRODUCTION

Osteosarcoma is a good model for the study of tissue-specific somatic mutations because the molecular genetics of osteosarcoma have been at least partially elucidated (1). Although osteosarcoma has no commonly associated benign precursors, previous data from several laboratories, including ours, have demonstrated that several genetic events are required for osteosarcoma formation (2–5). These studies have shown that homozygous alterations in both the *p53* and *RB1* genes occur in sporadic osteosarcoma. The importance of these genes in osteosarcoma has been confirmed by the discovery that germ-line mutations in either *RB1* or *p53* cause predisposition to osteosarcoma (6–10).

The question of what other genes are involved in osteosarcoma tumorigenesis can be addressed genetically. Additional genetic events that involve gene inactivation can be detected by using polymorphic loci from each chromosomal arm to assess the frequency that each chromosomal arm undergoes tumor-specific LOH[3] (11–13). Consistent or frequent LOH for all or part of a chromosomal arm in a tumor is a characteristic trait of tumor suppressor gene inactivation and a good predictor of the presence of a tumor suppressor gene (14–21).

Yamaguchi *et al.* (18) had previously examined osteosarcomas for genomic LOH. They found that in their group of osteosarcomas, as in other tumors, LOH for any single chromosome occurred in a fraction of the tumors, but when they compared frequency of LOH for each individual arm of the chromosomes separately, they found that the chromosomal arms that lost heterozygosity most frequently were 3q, 13q, 17p, and 18q. Two of those chromosomal arms had already been identified as containing genes important for osteosarcoma tumorigenesis; chromosome 13q is the location of the retinoblastoma susceptibility gene (*RB1*) at 13q14 (22, 23), whereas chromosome 17p is the location of the *p53* gene at 17p13 (24–26).

We chose to focus on chromosome 3q because no known tumor suppressor gene had been mapped to that chromosomal arm. To identify the region in which the tumor suppressor gene was located, we used a technique called mitotic mapping to sublocalize the region of LOH on the chromosomal arm (27). Mitotic mapping relies on the tendency of LOH to occur either by subchromosomal deletion or by mitotic recombination rather than by loss of the entire chromosome. By examining a large number of tumors for LOH, it is possible to localize the smallest region of LOH. This region should contain the tumor suppressor locus. We have identified a subchromosomal region of chromosome 3q between *D3S1212* and *D3S1246* that most likely contains the putative tumor suppressor gene. This region is less than 1 cM in genetic distance.

MATERIALS AND METHODS

Osteosarcoma samples were obtained from the primary or metastatic tumor sites in 41 patients. Osteosarcoma tumors were snap-frozen at −80°C, and DNA extraction was done as previously described (6). Matched normal DNA was extracted either from adjacent normal tissue or from peripheral blood samples, as described previously (28). This research was performed under approval by the Institutional Review Board for Human Subjects, and in all cases, informed consent was obtained from the patient or guardian prior to sample acquisition.

Polymorphic microsatellite loci were used to determine LOH. The loci used were *RHO* (29), *ACPP* (30), *D3S1509* (31), *D3S1282* (32), *D3S1212* (31), *D3S1246* (31), *SLC2A2* (33), and *SST* (29). All have been localized to the chromosome 3 genetic map (34). PCR amplifications were carried out in 50-μl volumes following the condition recommended. The samples were labeled by incorporation of [α-^{32}P]dCTP during the PCR amplification reaction. Five μl of each PCR reaction were diluted with 2 μl of bromphenol blue:Ficoll buffer (35) and separated by electrophoresis through 21 × 40-cm 7% polyacrylamide 1× TBE gels at 1.5 W overnight at room temperature. The gels were then exposed to X-ray film for 12–48 h at −80°C.

In several cases, the samples were not labeled by incorporation of [α-^{32}P]dCTP during the PCR amplification reaction. In those samples, the polymorphic alleles were viewed by staining with ethidium bromide following electrophoresis.

RESULTS

Matched normal and tumor DNA samples were initially screened for LOH using *RHO, ACPP, SLC2A2,* and *SST*. These markers span an approximately 77-cM region between chromosome 3q21.3-3q28. We found 12 tumors that showed no LOH for the entire region and 13 tumors that showed LOH for the entire region. The remaining 16 tumors showed tumor-specific mitotic recombination or deletion with at least one breakpoint that occurred within this region. The smallest region of common LOH for these tumors was between *ACPP* and *SLC2A2*. To further localize the region of LOH that most likely contained the putative tumor suppressor, we screened 13 matched sets of normal/tumor DNAs from those whose tumors had showed tumor-specific mitotic recombination or deletion breakpoints between *ACPP* and *SLC2A2*. We used four loci that mapped between *ACPP* and

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[3] The abbreviations used are: LOH, loss of constitutional heterozygosity; BDLS, Brachmann-de Lange syndrome.

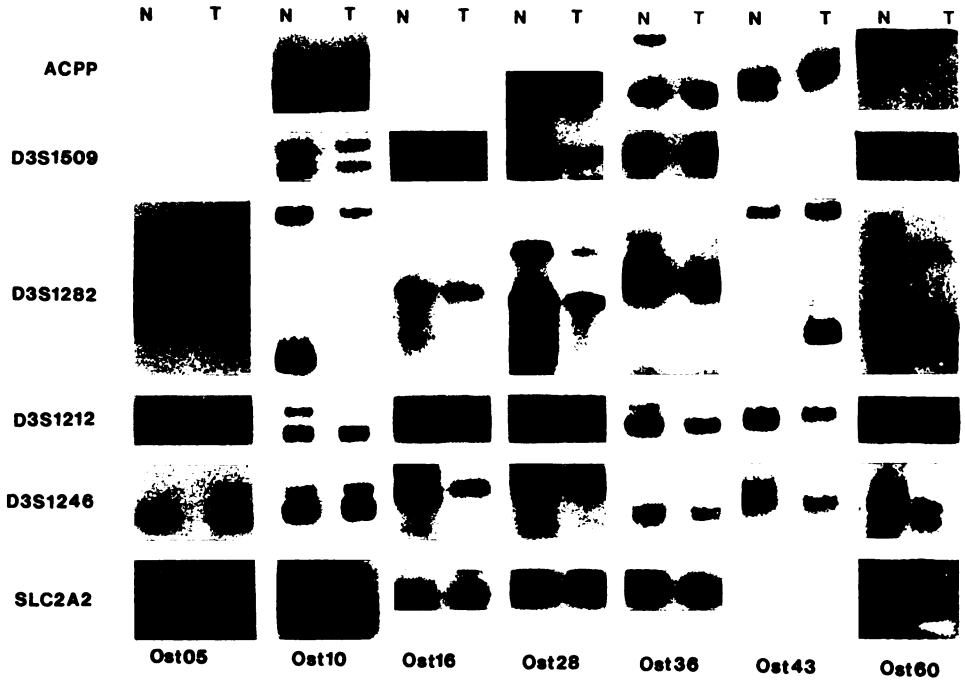

Fig. 1. Representative LOH analysis of chromosome 3q loci in osteosarcoma tumors using short tandem repeat polymorphisms. Matched normal (*N*) and tumor (*T*) samples from seven patients were amplified by PCR using primers that amplify each specific polymorphic locus. The loci are oriented centromeric to telomeric from *top* to *bottom* in the figure.

SLC2A2: *D3S1509, D3S1282, D3S1212,* and *D3S1246*. Fig. 1 shows representative examples of the results from the LOH analysis. In three cases (OST 01, 16, and 43), LOH occurred distal to *D3S1282*. In two cases (OST 05 and 19), LOH occurred distal to *D3S1212*. In one case (OST 46), LOH occurred proximal to *SST*. In another case (OST 61), LOH occurred proximal to *SLC2A2*. In three cases (OST 28, 36, and 60), LOH occurred proximal to *D3S1246*. In two cases (OST 22 and 39), the end points of the region of LOH could not be accurately determined because the normal DNA samples were uninformative at the critical loci *D3S1212* and *D3S1246*. However, the LOH data from these two sample sets were not inconsistent with LOH for the region including *D3S1212* and *D3S1246*. In one tumor sample (OST 10), we found what was apparently a deletion on one chromosome or a double mitotic recombination. The breakpoints of either the deletion or the recombination were between *D3S1509* and *D3S1282* on the proximal end and between *D3S1212* and *D3S1246* on the distal end such that only the region between *D3S1509* and *D3S1246* appeared to undergo LOH. Fig. 2 summarizes the data that we obtained for these samples.

DISCUSSION

The results reported here confirm that chromosomal alterations that lead to LOH on chromosome 3q are relatively common in osteosarcoma. We observed an overall LOH frequency of 70.7% (29 of 41). Although great care was taken to isolate DNA from homogeneous samples of tumor, contamination of the tumor tissue with normal tissue was apparent in some of the tumors, as can be seen in Fig. 1. We compared ratios of allelic intensity, and only those in which the allelic ratio was between 0.00 and 0.25 were taken to indicate LOH. Thus, these data may be considered a conservative estimate of LOH for 3q in osteosarcoma. Our finding of 70.7% LOH for chromosome 3q is in accordance with results reported previously (18).

The current model for mapping tumor suppressor genes is that the tumor suppressor gene will be located within the smallest region of LOH that is common to all tumor samples that undergo LOH. We screened 41 osteosarcoma patients using eight polymorphic loci on the long arm of chromosome 3q. We found 12 tumors that showed no LOH for the entire region tested (roughly 3q21.3-3q28) and 13 tumors that showed LOH for the entire region. The remaining 16 tumors showed tumor-specific mitotic recombination or deletion with at least one breakpoint that occurred within this region.

Of the matched samples that underwent LOH for only part of chromosome 3q, all had at least one breakpoint between *ACPP* and *SLC2A2*. As shown in Fig. 3, when the LOH data for these tumors were plotted on a chromosome 3q map, the data suggested a region of

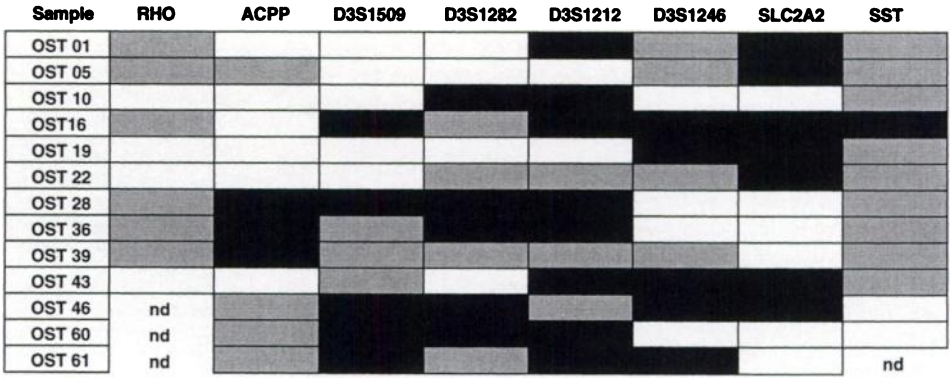

Fig. 2. Summary of LOH analysis of osteosarcoma samples with mitotic recombinations that occur within the *ACPP-SLC2A2* region. □, no LOH; ■, LOH; ▨, not informative; *nd*, not done.

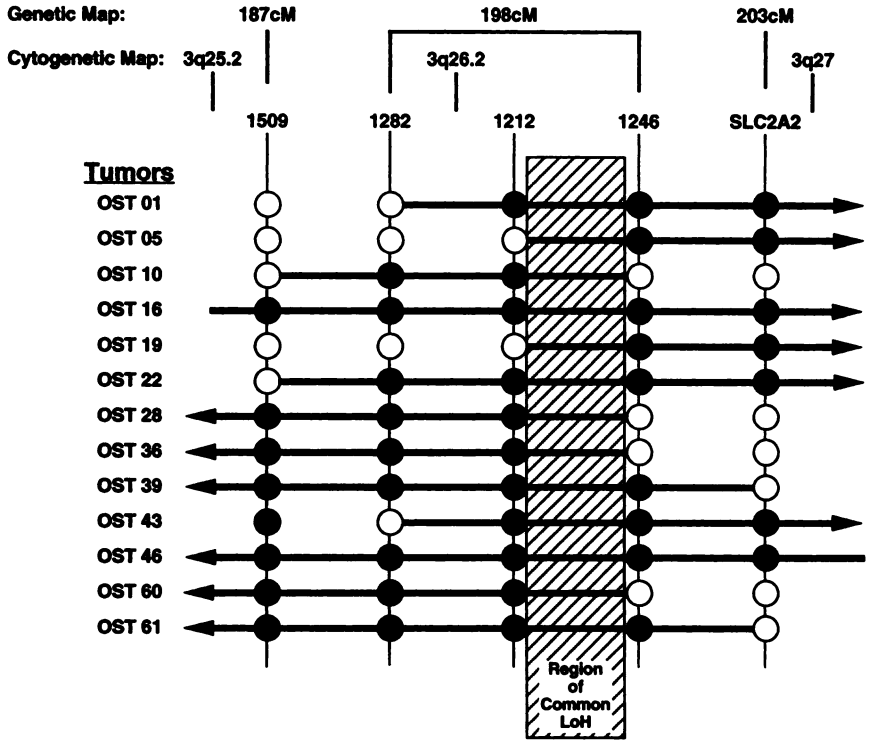

Fig. 3. Schematic map of the smallest common region of LOH from chromosome 3q in osteosarcoma. *Arrows*, regions of LOH in each of the tumors. The smallest region of common LOH is noted with a *hatched box*. Genetic distance markers in centimorgans and cytogenetic locations as defined by translocations are shown at the *top* of the figure [genetic distances and cytogenetic translocations are from Smith *et al.* (34)]. O, no LOH; ●, LOH; ⊙, not informative.

common LOH bounded proximally by *D3S1212* and distally by *D3S1246*. Thus, the putative tumor suppressor gene should lie between *D3S1212* and *D3S1246*. This region, which is located at 3q26.2–q26.3, represents a genetic distance of less than 1 cM because both *D3S1212* and *D3S1246* have been mapped at 198 cM on the chromosome 3 genetic map (34).

Several inferences can be made about the characteristics of the putative tumor suppressor located within this region. The percentage of LOH observed for chromosome 3q was comparable to the frequency of LOH seen at *RB1* or *p53*, two genes believed to be important in the initiation of osteosarcoma. This suggested that the tumor suppressor gene on chromosome 3q may also play a significant role in osteosarcoma tumorigenesis.

There is other evidence that suggests a gene important in bone development is located within this region. A dysmorphological syndrome called BDLS has also been mapped to this region of chromosome 3q (36, 37). BDLS is a disorder with a characteristic phenotype that includes prenatal and postnatal growth retardation, mental retardation, microcephaly, limb and digital anomalies, delayed skeletal maturation, abnormal thoracic configuration, and flat acetabular angles in infancy (38). The unusual radiological manifestations are related primarily to the limb anomalies, and these are often asymmetric. The phenotype is highly variable and evolves with age of the patient (39). The estimated frequency of BDLS is 0.6 per 100,000 with the great majority of cases of cases being sporadic (40). There are, however, a few reports of familial cases and of both concordant and discordant monozygotic twins (41–43).

The region to which predisposition to BDLS has been mapped overlaps with the smallest region of LOH to which we have mapped our osteosarcoma tumor suppressor gene. This raises the possibility that both the dysmorphology and tumorigenic predisposition may be due to alterations in the same gene. Previous studies have shown dysmorphology syndromes to be associated with tumor suppressor genes. One association is as part of a contiguous gene syndrome such as the WAGR syndrome in which deletion of a number of genes, including the Wilms' tumor locus *WT1*, occurs (44, 45). Alternatively, different types of genetic alterations in the same gene can lead to either dysmorphology or tumorigenesis. Mutations in the *PAX3* gene have been shown to result in Waardenburg's syndrome (46–50), whereas translocations involving the *PAX3* gene to the *FRKHD* gene are involved in alveolar rhabdomyosarcoma tumorigenesis (51, 52). It is thus possible to speculate that BDLS, a disease involving bone dysmorphology, may be linked to the 3q tumor suppressor locus, either directly in that the same gene causes both diseases, or the two genes may represent parts of a contiguous gene syndrome.

The localization of a possible tumor suppressor gene that is important in osteosarcoma tumorigenesis to a region that may be involved in other bone-related diseases points up the potential association between bone development and neoplasia. The isolation of this gene from the relatively small region of chromosome 3q will be an important step in the understanding of osteosarcoma tumorigenesis and may possibly be important in understanding regulation of bone development as well.

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REFERENCES

1. Kruzelock, R. P., and Hansen, M. F. Molecular genetics and cytogenetics of sarcomas. Hematol.-Oncol. Clin. N. Am., *9:* 513–540, 1995.
2. Hansen, M. F. Molecular genetic considerations in osteosarcoma. Clin. Orthop. Relat. Res., *270:* 237–246, 1991.
3. Scheffer, H., Kruize, Y. C., Osinga, J., Kuiken, G., Oosterhuis, J. W., Leeuw, J. A., Schraffordt-Koops, H., and Buys, C. H. Complete association of loss of heterozygosity of chromosomes 13 and 17 in osteosarcoma. Cancer Genet. Cytogenet., *53:* 45–55, 1991.
4. Toguchida, J., Ishizaki, K., Sasaki, M. S., Ikenaga, M., Sugimoto, M., Kotoura, Y., and Yamamuro, T. Chromosomal reorganization for the expression of recessive mutation of retinoblastoma susceptibility gene in the development of osteosarcoma. Cancer Res., *48:* 3939–3943, 1988.
5. Toguchida, J., Ishizaki, K., Nakamura, Y., Sasaki, M. S., Ikenaga, M., Kato, M.,

Sugimoto, M., Kotoura, Y., and Yamamuro, T. Assignment of common allele loss in osteosarcoma to the subregion 17p13. Cancer Res., *49:* 6247–6251, 1989.
6. Hansen, M. F., Koufos, A., Gallie, B. L., Phillips, R. A., Fodstad, O., Brogger, A., Gedde-Dahl, T., and Cavenee, W. K. Osteosarcoma and retinoblastoma: a shared chromosomal mechanism revealing recessive predisposition. Proc. Natl. Acad. Sci. USA, *82:* 6216–6220, 1985.
7. Srivastava, S., Zou, Z. Q., Pirollo, K., Blattner, W., and Chang, E. H. Germ-line transmission of a mutated *p53* gene in a cancer-prone family with Li-Fraumeni syndrome. Nature (Lond.), *348:* 747–749, 1990.
8. Iavarone, A., Matthay, K. K., Steinkirchner, T. M., and Israel, M. A. Germ-line and somatic *p53* gene mutations in multifocal osteogenic sarcoma. Proc. Natl. Acad. Sci. USA, *89:* 4207–4209, 1992.
9. McIntyre, J. F., Smith-Sorensen, B., Friend, S. H., Kassell, J., Borresen, A. L., Yan, Y. X., Russo, C., Sato, J., Barbier, N., Miser, J., Malkin, D., and Gebhardt, M. C. Germline mutations of the *p53* tumor suppressor gene in children with osteosarcoma. J. Clin. Oncol., *12:* 925–930, 1994.
10. Luca, J. W., Strong, L. C., and Hansen, M. F. A germline missense mutation in exon 10 of the human *p53* gene. Hum. Mut., in press, 1996.
11. Cavenee, W. K., Dryja, T. P., Phillips, R. A., Benedict, W. F., Godbout, R., Gallie, B. L., Murphree, A. L., Strong, L. C., and White, R. L. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature (Lond.), *305:* 779–784, 1983.
12. Hansen, M. F., and Cavenee, W. K. Genetics of cancer predisposition. Cancer Res., *47:* 5518–5527, 1987.
13. Hansen, M. F., and Cavenee, W. K. Tumor suppressors: recessive mutations that lead to cancer. Cell, *53:* 173–174, 1988.
14. Vogelstein, B., Fearon, E. R., Kern, S. E., Hamilton, S. R., Preisinger, A. C., Nakamura, Y., and White, R. Allelotype of colorectal carcinomas. Science (Washington DC), *244:* 207–211, 1989.
15. Sato, T., Saito, H., Morita, R., Koi, S., Lee, J. H., and Nakamura, Y. Allelotype of human ovarian cancer. Cancer Res., *51:* 5118–5122, 1991.
16. Knowles, M. A., Elder, P. A., Williamson, M., Cairns, J. P., Shaw, M. E., and Law, M. G. Allelotype of human bladder cancer. Cancer Res., *54:* 531–538, 1994.
17. Mitra, A. B., Murty, V. V., Li, R. G., Pratap, M., Luthra, U. K., and Chaganti, R. S. Allelotype analysis of cervical carcinoma. Cancer Res., *54:* 4481–4487, 1994.
18. Yamaguchi, T., Toguchida, J., Yamamuro, T., Kotoura, Y., Takada, N., Kawaguchi, N., Kaneko, Y., Nakamura, Y., Sasaki, M., and Ishizaki, K. Allelotype analysis in osteosarcomas: frequent allele loss on 3q, 13q, 17p, and 18q. Cancer Res., *52:* 2419–2423, 1992.
19. Seymour, A. B., Hruban, R. H., Redston, M., Caldas, C., Powell, S. M., Kinzler, K. W., Yeo, C. J., and Kern, S. E. Allelotype of pancreatic adenocarcinoma. Cancer Res., *54:* 2761–2764, 1994.
20. Aoki, T., Mori, T., Du, X., Nisihira, T., Matsubara, T., and Nakamura, Y. Allelotype study of esophageal carcinoma. Genes Chromosomes Cancer, *10:* 177–182, 1994.
21. Nawroz, H., van der Riet, P., Hruban, R. H., Koch, W., Ruppert, J. M., and Sidransky, D. Allelotype of head and neck squamous cell carcinoma. Cancer Res., *54:* 1152–1155, 1994.
22. Sparkes, R. S., Sparkes, M. C., Wilson, M. G., Towner, J. W., Benedict, W. F., Murphree, A. L., and Yunis, J. J. Regional assignment of genes for human esterase D and retinoblastoma to chromosome band 13q14. Science (Washington DC), *208:* 1042–1044, 1980.
23. Higgins, M. J., Hansen, M. F., Cavenee, W. K., and Lalande, M. Molecular detection of chromosomal translocations that disrupt the putative retinoblastoma susceptibility locus. Mol. Cell. Biol., *9:* 1–5, 1989.
24. Isobe, M., Emanuel, B. S., Givol, D., Oren, M., and Croce, C. M. Localization of gene for human p53 tumour antigen to band 17p13. Nature (Lond.), *320:* 84–85, 1986.
25. McBride, O. W., Merry, D., and Givol, D. The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). Proc. Natl. Acad. Sci. USA, *83:* 130–134, 1986.
26. Miller, C., Mohandas, T., Wolf, D., Prokocimer, M., Rotter, V., and Koeffler, H. P. Human *p53* gene localized to short arm of chromosome 17. Nature (Lond.), *319:* 783–784, 1986.
27. Scrable, H. J., Witte, D. P., Lampkin, B. C., and Cavenee, W. K. Chromosomal localization of the human rhabdomyosarcoma locus by mitotic recombination mapping. Nature (Lond.), *329:* 645–647, 1987.
28. Miller, S. A., Dykes, D. D., and Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res., *16:* 1215, 1988.
29. Weber, J. L., and May, P. E. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Genet., *44:* 388–396, 1989.
30. Doak, S., Jordan, S., McWilliam, P., and Humphries, P. Tetranucleotide repeat polymorphism at the *ACPP* locus. Nucleic Acids Res., *19:* 4793, 1991.
31. Naylor, S. L., Buys, C. H., and Carritt, B. Report and abstracts of the Fourth International Workshop on Human Chromosome 3 Mapping. Cytogenet. Cell Genet., *65:* 2–50, 1994.
32. Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., and Weissenbach, J. The 1993–94 Genethon human genetic linkage map. Nat. Genet., *7:* 246–339, 1994.
33. Granqvist, M., Xiang, K., Seino, M., Fukumoto, H., and Bell, G. I. Dinucleotide repeat polymorphism in human GLUT2/liver facilitative glucose transporter gene on chromosome 3. Nucleic Acids Res., *19:* 4791, 1991.
34. Smith, D. I., Glover, T. W., Gemmill, R., Drabkin, H., O'Connell, P., and Naylor, S. L. Report of the Fifth International Workshop on Human Chromosome 3 Mapping 1994. Cytogenet. Cell Genet., *68:* 126–146, 1995.
35. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual, Ed. 2, pp. 6.12–6.45. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
36. Ireland, M., English, C., Cross, I., Houlsby, W. T., and Burn, J. A *de novo* translocation t(3;17)(q26.3;q23.1) in a child with Cornelia de Lange syndrome. J. Med. Genet., *28:* 639–640, 1991.
37. Holder, S. E., Grimsley, L. M., Palmer, R. W., Butler, L. J., and Baraitser, M. Partial trisomy 3q causing mild Cornelia de Lange phenotype. J. Med. Genet., *31:* 150–152, 1994.
38. Braddock, S. R., Lachman, R. S., Stoppenhagen, C. C., Carey, J. C., Ireland, M., Moeschler, J. B., Cunniff, C., and Graham, J. M., Jr. Radiological features in Brachmann-de Lange syndrome. Am. J. Med. Genet., *47:* 1006–1013, 1993.
39. Ireland, M., Donnai, D., and Burn, J. Brachmann-de Lange syndrome: delineation of the clinical phenotype. Am. J. Med. Genet., *47:* 959–964, 1993.
40. Beck, B. Epidemiology of Cornelia de Lange's syndrome. Acta Paediat. Scand., *65:* 631–638, 1976.
41. Bankier, A., Haan, E., and Birrell, R. Familial occurrence of Brachmann-de Lange syndrome. Am. J. Med. Genet., *25:* 163–165, 1986.
42. Robinson, L. K., Wolfsberg, E., and Jones, K. L., Brachmann-de Lange syndrome: evidence for autosomal dominant inheritance. Am. J. Med. Genet., *22:* 109–115, 1985.
43. Feingold, M., and Lin, A. E., Familial Brachmann-de Lange syndrome: further evidence for autosomal dominant inheritance and review of the literature. Am. J. Med. Genet., *47:* 1064–1067, 1993.
44. Bickmore, W. A., and Hastie, N. D., Aniridia, Wilms' tumor and human chromosome 11. Ophthalmic Paediat. Genet., *10:* 229–248, 1989.
45. Mannens, M., Hoovers, J. M., Bleeker-Wagemakers, E. M., Redeker, E., Bliek, J., Overbeeke-Melkert, M., Saunders, G., Williams, B., van Heyningen, V., Junien, C., and Hastie, N. The distal region of 11p13 and associated genetic diseases. Genomics, *11:* 284–293, 1991.
46. Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P., and Strachan, T. Waardenburg's syndrome patients have mutations in the human homologue of the *Pax-3* paired box gene. Nature (Lond.), *355:* 635–636, 1992.
47. Baldwin, C. T., Hoth, C. F., Amos, J. A., da-Silva, E. O., and Milunsky, A. An exonic mutation in the HuP2 paired domain gene causes Waardenburg's syndrome. Nature (Lond.), *355:* 637–638, 1992.
48. Hoth, C. F., Milunsky, A., Lipsky, N., Sheffer, R., Clarren, S. K., and Baldwin, C. T. Mutations in the paired domain of the human *PAX3* gene cause Klein-Waardenburg syndrome (WS-III) as well as Waardenburg syndrome type I (WS-I). Am. J. Hum. Genet., *52:* 455–462, 1993.
49. Tassabehji, M., Read, A. P., Newton, V. E., Patton, M., Gruss, P., Harris, R., and Strachan, T. Mutations in the *PAX3* gene causing Waardenburg syndrome type 1 and type 2. Nat. Genet., *3:* 26–30, 1993.
50. Baldwin, C. T., Lipsky, N. R., Hoth, C. F., Cohen, T., Mamuya, W., and Milunsky, A. Mutations in PAX3 associated with Waardenburg syndrome type I. Hum. Mut., *3:* 205–211, 1994.
51. Barr, F. G., Galili, N., Holick, J., Biegel, J. A., Rovera, G., and Emanuel, B. S. Rearrangement of the *PAX3* paired box gene in the paediatric solid tumour alveolar rhabdomyosarcoma. Nat. Genet., *3:* 113–117, 1993.
52. Galili, N., Davis, R. J., Fredericks, W. J., Mukhopadhyay, S., Rauscher, F. J. d., Emanuel, B. S., Rovera, G., and Barr, F. G. Fusion of a fork head domain gene to *PAX3* in the solid tumour alveolar rhabdomyosarcoma. Nat. Genet., *5:* 230–235, 1993.